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THE UNIVERSITY OF ALBERTA

A STUDY OF METHODS FOR MEASUREMENT OF FREE METAL ION CONCENTRATION IN SOLUTION

by -

IQBAL M TABANI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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EDMONTON, ALBERTA

SPRING 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A STUDY OF METHODS FOR MEASUREMENT OF FREE METAL ION CONCENTRATIONS IN SOLUTIONS submitted by IQBAL MOHAMMED TABANI in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Free metal ion concentrations in solution have been measured in this thesis by spectrophotometric and by ion exchange-atomic spectroscopic techniques. The first technique was used to measure ionic calcium concentrations with the indicator ligand arsenazo III. Protonation constants for arsenazo III and the conditional stability constants of the calcium arsenazo III complexes at pH 7 to 7.3 were determined. A previously proposed method for the estimation of free metal ion concentrations that corrects for indicator complexation, the sample ion increment method, was employed to determine calcium ion concentrations in synthetic solutions containing calcium plus various calcium complexing ligands. Preliminary measurements on urine samples indicated that the method shows promise for the measurement of ionic calcium in urine.

The sample ion increment method was also studied for the measurement of iron(III) in blood serum using xylenol orange as indicator. Conditional protonation constants for the ligand and the iron(III) ligand complex were measured at pH 1.25. The method when applied to several serum samples gave results that compared favorably to expected values.

A microcomputer-controlled data acquisition and

processing system was set up for an ion-exchange atomic-absorption instrument for metal ion speciation. The automated operations are to equilibrate a micro ion-exchange column with sample solution, wash the column, and elute the metal on the column into the atomic absorption spectrophotometer with nitric acid. Both software and hardware were developed to control all steps of data acquisition and processing. The instrument was tested for free calcium ion determinations in solutions containing various synthetic combinations of free and bound calcium. The experimental results agreed well with those calculated from equilibrium constant data.

TABLE OF CONTENTS

CHAPTER PAGE

PART 1

1. INTRODUCTION

1.1 Need to be Able to Measure Ionic Concentration of Calcium and Iron (111)..... 1

1.2 Methods Available for the Measurement of Ionic Concentrations of Calcium and Iron (111) in Biological Systems and their Limitations..... 5

1.3 Need for a Better Technique for the Measurement of Ionic Calcium and Iron (111) Concentrations in Biological Fluids..... 7

2. MEASUREMENT OF IONIC CALCIUM CONCENTRATIONS BY THE SAMPLE ION INCREMENT METHOD USING ARSENAZO-111

2.1 General Principle of the Sample Ion Increment Method..... 9

2.1.1. Model for free metal ion determination by spectrophotometry..... 9

2.1.2. Application of the model to conventional spectrophotometric methods..... 11

2.1.3. Quantitative considerations of metal ion buffering..... 13

2.1.4. Quantitative aspects of the conventional spectrophotometric methods..... 16

2.1.5. A modified spectrophotometric method: The Sample Ion Increment Method (SIIM)..... 20

2.2 Selection of a Metallochromic Indicator for the Determination of Ionic Calcium Concentrations..... 24

2.2.1 The choice of the indicator molecule and the SIIM requirements for indicators..... 26

2.3 Determination of Protonation Constants of Arsenazo - 111 and Conditional Formation Constants of the Calcium Arsenazo-111 System..... 30

2.3.1. Experimental..... 30

2.3.2. Aparatus..... 31

2.3.3 Procedure..... 32

2.4 Conditional Formation Constants of Calcium Arsenazo-III Complexes..... 33

2.5 Results and Discussion..... 34

2.6 Effect of Proton Dissociation on the Spectrum of Arsenazo-III..... 44

2.7 Effect of Calcium on the Spectrum of Arsenazo-III..... 46

2.8 Determination of Free Calcium Concentrations in Synthetic Samples by SIIM..... 51

2.8.1. Preparation of the synthetic samples and measurement of free concentration of calcium by SIIM..... 53

2.9 Preliminary Measurement of Free Calcium Concentrations in Urine Samples by SIIM..... 54

3. MEASUREMENT OF IONIC CONCENTRATIONS OF IRON (III) BY THE SAMPLE ION INCREMENT METHOD USING XYLENOL-ORANGE

3.1 Introduction..... 63

3.2 Determination of Reaction Stoichiometry and Conditional Formation Constants for the Iron (III) Xylenol-Orange Complex..... 64

3.2.1. Experimental..... 64

3.2.2. Optimization of the experimental conditions for the spectrophotometric measurement of Fe (III) xylenol-orange complex..... 65

3.2.3. Titration apparatus and procedure..... 66

3.3 Discussion..... 68

3.4 Determination of Free Iron (III) in Synthetic Samples by SIIM..... 74

3.5 Determination of Non-Heme Iron (III) in Blood Serum by SIIM..... 79

3.5.1. Experimental procedure..... 82

4. MICROCOMPUTER CONTROLLED DATA-ACQUISITION/PROCESSING OF AN ION EXCHANGE ATOMIC-ABSORPTION SYSTEM FOR DETERMINATION OF FREE METAL ION CONCENTRATIONS

4.1 Theory of Ion Exchange Equilibration Method..... 84

4.2 Need for Automation of the Ion Exchange Equilibration Technique..... 86

4.2.1 Introduction..... 88
4.3 Overall System Design..... 89
4.3.1. Hardware modules..... 92
4.3.2. Software modules..... 98
4.4 Evaluation of System..... 101

5. MEASUREMENT OF FREE CALCIUM CONCENTRATIONS BY ION - EXCHANGE EQUILIBRATION/ATOMIC-ABSORPTION SPECTROSCOPY (IEE-AAS)

5.1 Experimental..... 112
5.2 Characterization of IEE-AAS Technique..... 113
5.3 Determination of Free Calcium Concentrations in Synthetic Samples by IEE-AAS..... 118
5.4 Discussion..... 130
5.5 Determination of Free Calcium Concentrations in Urine by IEE-AAS..... 136
5.5.1. Results and discussion..... 137

REFERENCES..... 141-150

LIST OF TABLES

TABLE		PAGE
2.1.	Computer Calculated Values of $(\partial[M]/\partial F'M)K'L,FL$ as a Function of F'M, K'L, and FL	17
2.2.	Properties of Metallochromic Indicators for Calcium	27
2.3.	Stability Constants of Calcium Arsenazo-III Complexes	28
2.4.	Protonation Constants of Arsenazo-III	42
2.5.	Conditional Stability Constants of Calcium Arsenazo - III Complexes	50
2.6.	Summary of the Scheme for the Preparation of Synthetic Samples	55
2.7.	Free Calcium Determination by SIIM in Synthetic Sample	56
2.8.	Free Calcium Determination by SIIM in Synthetic Sample	57
2.9.	Free Calcium Determination by SIIM in Synthetic Sample	58
2.10.	Free Calcium Determination by SIIM in Synthetic Sample	59
2.11.	Summary of Free Calcium Concentration Obtained in Urine Samples by SIIM	62
3.1.	Comparison of Calculated and Experimental Values of Free Iron (III) Obtained by the Sample Ion Increment Method	77
3.2.	Comparison of Calculated and Experimental Values of Free Iron (III) Obtained by the Sample Ion Increment Method	78
3.3.	Comparison of Free Iron (III) Concentrations Obtained by Single measurement and the Sample Increment Method	80
5.1.	Effect of Ionic Strength on Equilibration Time	119
5.2.	Scheme for the Preparation of Synthetic Samples	121
5.3.	Comparison of Ion-Exchange Equilibration/Atomic-absorption and Ion Selective Electrode for the Determination of Ionic Concentration of Calcium in Urine Samples	139

LIST OF FIGURES

Figure	Description	Page
2.1	Model for the determination of free metal ion concentrations	10
2.2	Relation between free metal ion concentration [M] and F'_M at various levels of metal ion buffering	18
2.3	Expanded section of a curve from figure 2.2 illustrating the single measurement method	21
2.4	Expanded section of a curve from figure 2.2 illustrating the sample ion increment method	22
2,5	Visible absorption spectrum of arsenazo-111, calcium arsenazo-111 and magnesium arsenazo-111 complexes	29
2.6	pH titration of 2.5 X 10^{-5}M arsenazo-111 in 0.1M KNO_3 with 7.50 X 10^{-3}M NaOH. Experimental points indicated by; solid lines generated by computer using Miniquad81 calculated protonation constants.	43
2.7	Visible absorption spectrum of 7.6 X 10^{-5}M arsenazo-111 in 0.1M KNO_3 as a function of pH	45
2.8	Fraction of arsenazo-111 present in various ionic forms as a function of pH, calculated by COMIX	47
2.9	Visible absorption spectrum of 2:1 calcium-arsenazo-111 solution in 0.1M KNO_3 as a function of pH. Concentration of arsenazo-111 7.6 X 10^{-5}M	48
2.10	Visible absorption spectrum of 2.55 X 10^{-5}M calcium arsenazo-111 in 0.1M KNO_3 at pH 12.1	52
2.11 (a)	Visible absorption spectrum of urine	61

(b) Visible absorption spectrum of calcium-arsenazo-III complex in urine..... 61

3.1 Visible absorption spectrum of 2.5 X 10^{-5} M iron (III) xylenol orange at pH 1.2 and ionic strength 0.1 in KNO_3..... 67

3.2 Effect of pH on the absorption of iron (III) xylenol orange complex. Concentration of iron (III) 2.00 X 10^{-4}M and xylenol orange 2.75 X 10^{-5}M..... 69

3.3 (a) Photometric titration of 2.15 X 10^{-5} M xylenol orange with 2.0 X 10^{-4}M iron (III) at pH 1.25 and ionic strength of 0.1 in KNO_3..... 70

(b) Photometric titration of 2.15 X 10^{-5} M xylenol orange with 2.0 X 10^{-4}M iron (III) at pH 1.25 and ionic strength of 0.1 in KNO_3..... 71

3.4 Job's Plot..... 72

3.5 Frank and Oswald plot for the determination of conditional formation constants of iron (III) -xylenol orange complex..... 75

4.1 Flow chart of the semi-automated ion-exchange atomic absorption spectroscopy system for free metal ion determination. System prior to automation..... 87

4.2 Design cycle for a microcomputer based automated laboratory instrument system..... 90

4.3 Schematic of the interface board DT-2805..... 95

4.4 (a) Circuit diagram of signal conditioning section of valve controller..... 96

(b) Circuit diagram of valve driver section of valve controller..... 97

4.5 Overall integrated (ion-exchange atomic absorption) hardware system..... 99

4.6 Modularization of software units for automated ion-exchange atomic absorption spectroscopy..... 100

4.7 Flow chart for control cycle for valve control logic and data-acquisition..... 102

4.8 Flow chart for control cycle for timing and plotting..... 103

4.9 Schematic for valve control logic and positioning during different cycles. See figure 4.5 for identification of valves..... 104

4.10 Main manual; options available..... 106

4.11 Experimental setup record for IEE-AAS..... 107

4.12 Setup for initialization of solenoid valves..... 109

4.13 (a) Calibration plot for free calcium, peak height against concentration..... 110

(b) Calibration plot for free calcium, peak area against concentration..... 111

5.1 Relative sensitivity of the AA signal at various flow rates of solution into the nebulizer..... 114

5.2 Equilibration time required for columns of different sizes..... 116

5.3 Effect of varying pH on the uptake of calcium by resin..... 117

5.4 Combined effect of increased K+ and ionic strength on the uptake of calcium by resin..... 120

5.5 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in presence of citrate..... 122

5.6 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-citrate system..... 124

5.7 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in presence of tartrate..... 125

5.8 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-tartrate system..... 126

5.9 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in the presence of phosphate... 128

5.10 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-phosphate system..... 129

5.11 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in the presence of oxalate..... 131

5.12 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-oxalate system..... 132

5.13 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in presence of NTA..... 133

5.14 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-NTA system..... 134

CHAPTER 1

INTRODUCTION

1.1 THE NEED TO BE ABLE TO MEASURE IONIC CONCENTRATIONS OF CALCIUM AND IRON (III)

Speciation is a concept of critical importance for evaluating the impact of metal ions on life. Speciation of an element is the determination of the individual physico-chemical forms of that element which together make up its total concentration in a sample. It is now well established that the measurement of the total elemental concentration is often inadequate for evaluating the toxicity of metal ions and for understanding the role of trace metals in various physiological systems. Additional information characterizing the molecular and physical forms of an element may be required to predict its toxicity and mobility. For transition and many heavy metals, for example, it is the free (hydrated) metal ion that is generally recognized to be the most toxic form (1-4).

Reports of metal speciation studies appear in the literature with increasing frequency. In some reports the term "speciation" refers only to the kinetically inert species present in a sample. This type of speciation can be accomplished by techniques such as filtration (5), solvent extraction (6), chromotagraphy (7), and some ion-exchange methods (8). All of these techniques distinguish only species that are not capable of rapid interconversion with one another; all interconverting kinetically labile species appear together as one species. In other reports "speciation" is used in a broader sense to also include a distinction among labile species. Techniques employing ion selective electrodes (9), indicator dyes (10-12), ion-exchange (13-17), and in some cases electrochemical techniques such as voltammetry and anodic stripping at selected potentials (18-19) can

be used to differentiate between labile species. The crucial importance of defining the speciation (physico-chemical forms) of metals in aquatic (fresh and sea water) and biological systems is now well established (20-23). Esentially three approaches have been applied to the study of metal speciation:

(1) the experimental determination of the interaction between metal ions and the various chemical components (i.e. ligands) in simplified model systems.

(2) the development of theoretical models based upon experimentally determined stability constants and thermodynamic considerations: and

(3) the determination of specific metal forms in an actual sample.

All three approaches require development in a complimentary fashion as alone none will provide a definitive answer. However, the first two approaches have proved far easier to apply and thus provide much of the information currently available. Thus there is now a clear need for improved experimental techniques to supplement the third approach, the determination of metal forms such as the free metal ion to validate existing methods, as well as highlight their weakness, and to indicate areas for further studies.

The analysis of biological fluids such as blood plasma, serum and urine has been used for many years as an aid in the diagnosis of certain diseases, and in biochemical and biophysical research. Calcium is an important element in biological systems and calcium balances are important in the investigations of metabolic bone disease and in studying the effects of antibotics, drugs and physiological substances, such as hormones, upon bone (24,25). Ionized calcium (free, hydrated calcium ion) is recognized as the physiologically active form of calcium. Calcium is present in the blood plasma in three different forms; ionized (free, hydrated ion), bound to protein and complexed with molecules such as lactate and citrate (26,27). The concentration of ionic calcium in blood serum better reflects changes in calcium homeostasis than does the total serum calcium. Chronic renal failure has been attributed to abnormal calcium metabolism and regular

monitoring of ionic serum calcium is therefore important (28-30). The diagnostic evaluation of patients with kidney stones often requires measurement of ionic calcium along with oxalate, phosphate and uric acid concentrations. Calcium oxalate mixed with calcium phosphates compose the largest group of renal calculi occuring in humans. In a large series of stones whose composition was carefully analyzed they accounted for 78% of the total while magnesium ammonium phosphates, usually mixed with other phosphates, accounted for another 16% and uric acid, urates and cystines represented the major fraction remaining (31,31).

Knowledge of the urinary excretion of calcium can be particularly important in patients with renal calculi because ionic calcium excretion is as high as 40% more than can be accounted for, and hence treatment may be predicated on the biochemical values (31,34). Measurement of free (ionic) calcium and magnesium is crucial in the bioclinical investigation of calcium and magnesium in the histones fraction of normal and tumor cells (35) and in studies of calcium binding to nucleic acids (36,37).

Iron (111) is an essential component of biological systems but, in common with all elements, becomes toxic if in excess. For iron (111), therefore, a fairly narrow "concentration window" exists between essential and toxic levels. Toxicity may arise from the precipitation of excess iron (111) in tissues or from a lack of sufficient transferrin (iron (111) transport protein) or ferritin (iron (111) storage protein) in biological fluids. The particular toxic role of iron (111) in including specific manifestations of organ failure associated with serious iron (111) overload is briefly summarized in the following sections. For a detailed discussion a recent review on iron overload should be consulted (38).

Iron Overload and the Liver: Hepatic fibrosis is common to all forms of iron overload and almost all subjects with idiopathic hemochromatosis

have cirrhosis by the time the condition becomes clinically manifest (39). In affected but preclinical siblings iron overload precedes the onset of cirrhosis (40,41). Serious postal fibrosis or cirrhosis has been found to be directly related to hepatic iron (111) concentration (42). The evidence that iron (111) is a fibrogenic agent and can cause hepatic cirrhosis appears overwhelming.

Iron Overload and the Pancreas: Diabetes is one of the most common clinical manifestations of idiopathic hemochromatosis; about 80% of idiopathic hemochromatosis patients have diabetes. Its occurrance in iron overload and transfusional hemochromatosis strengthens the concept that iron overload plays a part in its pathogenesis. Evidently heavy iron deposits in the islets cells of the pancreas lead to reduced insulin output and hence diabetes (43). Impaired glucose tolerance and diabetes mellitus are known complications of transfusional iron overload, and the mechanism of hepatic damage due to iron overload has been reported (44,45).

Iron Overload and the Heart: The most direct evidence of iron overload has been obtained for disorders of cardiac functions (39). Cardiac failure is suggested as the most common cause of death in idiopathic hemochromatosis; here the cardiopathy is more marked when the clinical disease materializes (43,46). Recently it has been reported that the concentration of iron (111) in serum decreases considerably during open heart surgery (47).

It has also been reported that ingestion of oral contraceptive drugs may lead to high levels of iron (111) in blood serum (48). Iron absorption via the diet is physiologically controlled; the body has no regulatory mechanism for eliminating a toxic excess introduced by accidential overdose or by multiple transfusion. Hence the ability to monitor free iron (111) concentrations allows administration of iron (111) chelating drugs to reduce iron overload.

1.2 METHODS AVAILABLE FOR THE MEASUREMENT OF IONIC CONCENTRATIONS OF CALCIUM AND IRON (111) IN BIOLOGICAL SYSTEMS AND THEIR LIMITATIONS:

The literature reveals that the measurement of ionic concentrations of calcium in biological fluids is mainly done either spectrophotometrically or potentiometrically. However, direct potentiometric methods employing calcium ion selective electrodes are often limited by lack of selectivity, difficulties in matching standards to samples, variations in ionic strength and uncertainties in junction potentials, especially if the measurement is being carried out on urine samples (49).

The spectrophotometric method involves the addition of a metallochromic indicator which forms a highly coloured but weak complex with, for example, calcium ions. The concentration of the calcium-indicator complex is measured spectrophotometrically. From this concentration, along with knowledge of the total ligand added and the conditional formation constants of the calcium-indicator complex, the free (ionic) calcium concentration is then calculated (50). Two modifications have been proposed to improve and extend the usefulness of the spectrophotometric method. The first allows use of some indicators of less than 100% purity by measuring the absorbance at two wavelengths; this technique has been discussed by Raaflaub (51) and used by several (52,53). The other involves measuring the difference between the absorbance of the solution at two wavelengths (54). This approach has been particularly useful when the indicator selectivity is poor (55).

As with other potentiometric measurements of free calcium ion concentrations, present spectrophotometric methods also have several limitations. The most severe restriction is that only a small fraction

of the ionic calcium, usually less than 1%, may be allowed to complex with the indicator if the equilibria existing in the original sample solution are not to be significantly preturbed. This requires a relatively small value for the conditional formation constant(s) of the calcium-indicator complex(es), which places severe limitations on the sensitivity of the method.

In addition to metallochromic indicators for the measurement of ionic calcium concentrations, fluormetric indicators have been proposed. These include Quin-2 (56) and chlorotetracycline (57,58). Quin-2 and chlorotetracycline have been used exclusively in the measurement of intracellular ionic calcium concentrations (59-61), but the use of these indicators in the measurement of ionic calcium concentrations in urine has not been reported because pH variations in urine influence the fluorescence of the calcium-indicator complex. The use of chlorotetracycline is also limited due to a high affinity for magnesium, which limits the selectivity. Also, the presence of detergents and other organic compounds enhance the fluorescence of the calcium-indicator complex, causing a serious interference in biological fluids, especially in urine (57,58). Tsien has proposed a new series of modified fluorescent indicators for ionic calcium determination. Quin-2AM, one of these indicators currently under investigation, is favoured by Tsien and other biochemists. Quin-2AM has been reported to be superior to other fluorescent indicators because the fluorescence of its calcium complex is not influenced by pH (62-65).

Another method of measuring the ionic concentration of calcium is the use of photoproteins which luminesce in the presence of calcium. Few photoproteins have been proposed for this purpose; they include aequorin (66-68), obelin (69,70), halistaurin (71), mnemiopsim and berovin (72); of these only aequorin seems to be used extensively (73-76), again for intracellular ionic calcium measurement. The greatest barrier to the use of photoproteins is undoubtedly the difficulty

encountered in isolating and purifying them.

At present, there is no method available which can measure the concentration of unbound (labile) iron (111) in the presence of the bound form in the biological fluids such as blood serum, in spite of the fact that iron (111) plays an important part in the physiological system (section 1.1). Part of the work in this thesis describes a modified spectrophotometric method (chapter 2) which reliably measures the concentration of labile iron (111) (not protein bound form) in the presence of the bound form in synthetic samples and in blood serum.

1.3 NEED FOR A BETTER TECHNIQUE FOR THE MEASUREMENT OF IONIC CALCIUM AND IRON (111) CONCENTRATIONS IN BIOLOGICAL FLUIDS:

As discussed in section 1.2, present spectrophotometric and potentiometric techniques for the measurement of ionic concentrations of calcium suffer from numerous limitations, and hence in order to evaluate ionic concentrations in physiological systems, more reliable and accurate methods are required. Moreover, in order to apply any technique in an environment such as hospitals or clinical laboratories as much of the methodology has to be automated if possible in order to increase accuracy, precision and economy. None of the speciation methods presently available have been completely automated.

Therefore part of this research work was concerned with the development of an automated technique to reliably measure the ionic concentration of calcium and iron (111) in biological fluids. This thesis has two sections corresponding to two different experimental approaches to determine ionic concentrations of calcium and iron (111). The first section deals with a modified spectrophotometric method, the Sample

Ion Increment Method (SIIM) (11), and its application to the determination of ionic calcium and iron (III) concentrations. The second section of this thesis is concerned with the application of the Ion-Exchange Equilibration Method (16) to the determination of free calcium concentrations and the development of a completely automated ion-exchange equilibration system designed around a microcomputer and its application in the determination of ionic calcium in urine, using atomic absorption as the detector.

CHAPTER 2

MEASUREMENT OF IONIC CALCIUM CONCENTRATIONS BY THE SAMPLE ION INCREMENT METHOD USING ARSENAZO-111

2.1 General Principle of the Sample Ion Increment Method:

A number of limitations inherent in the present spectrophotometric methods for the measurement of free metal ion concentration, as discussed in chapter 1, section 1.2, can be overcome by the approach described by Fulton and Kratochvil (11), the "Sample Ion Increment Method (SIIM)". In this modified spectrophotometric method, a known amount of indicator is added to the sample and the absorbance is measured. Then an accurately known amount of the sample ion to be determined is added and the absorbance is remeasured. The second measurement determines the sample ion buffering capacity of the original solution and permits a correction for the amount of sample ion complexed by the indicator. With this approach close to 100% of the sample ion can be complexed by the indicator without introducing appreciable determinate error, thereby reducing the previous stringent requirements on the magnitude of the conditional formation constant and the molar absorptivity of the sample ion-indicator complex. The background and derivation of this method are described in section 2.1. This discussion is based on published and unpublished work of Fulton (11).

2.1.1 Model For Free Metal Ion Determination By Spectrophotometry:

The concept behind the spectrophotometric measurement of free metal ion concentration in solution can be summarized by the equilibrium represented in figure 2.1. "M" is the free metal ion of interest which is originally in equilibrium with an auxilary ligand "L". The ligand L, which may undergo protonation, acts as a metal ion buffer for the metal M.

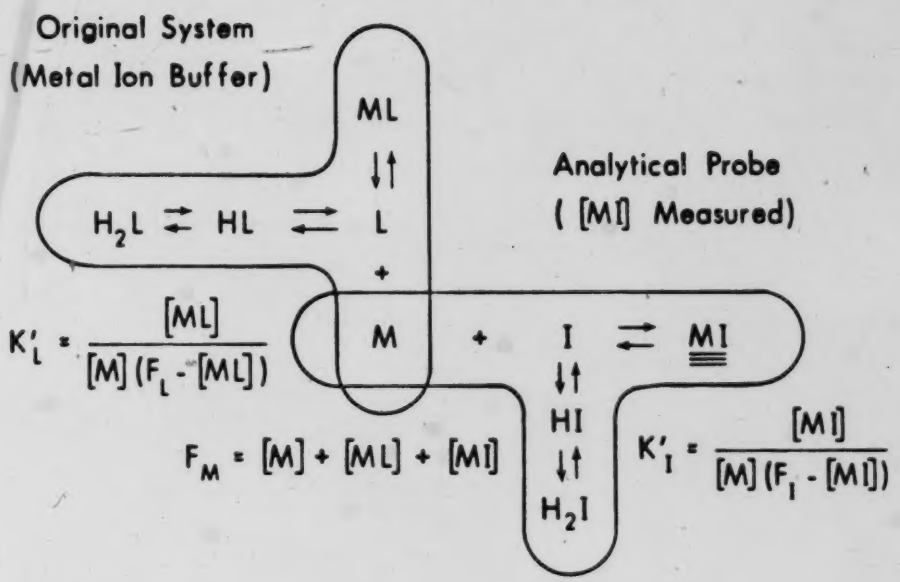


Figure 2.1 Model for the determination of free metal ion concentrations

F_M Total concentration of metal M

F_L Total concentration of the auxiliary ligand L

F_I Total concentration of the indicator ligand I

K'_L and K'_I Conditional formation constants for the metal-auxiliary and indicator ligands.

The degree of metal ion buffering is determined by the conditional formation constants for the metal ligand complex(es) and the equilibrium concentration of L. The model described here considers for convenience only a single dibasic auxiliary ligand forming one metal ligand complex, but more than one metal ligand complex can be accommodated easily. Moreover it is the concentration of the free metal ion, [M], that is sought, and not the details of how the metal ion is buffered. The analytical probe used to measure the ionic concentration of M is an indicator, I, added in such a way that dilution of the original sample solution is negligible. Again, for simplicity, the indicator is assumed to be dibasic, but protonation to any degree can be easily accommodated by the method. The indicator I forms a 1:1 coloured metal ligand complex, MI, which is measured spectrophotometrically. It is important that the addition of the indicator to the sample cause as little disruption to existing equilibria as possible. If the equilibrium concentration of I or the conditional formation constant of MI is too large, or if the pH of the original sample solution is changed upon addition of the indicator, the original equilibria will shift, introducing a determinate error. Generally the magnitude of this error is unknown, but because it can be large (see later discussion), conditions are usually selected such that only a small fraction, 1% or less, of the original free metal ion present complexes with the indicator. This greatly limits the sensitivity of the method.

2.1.2. Application of the Model to Conventional Spectrophotometric Methods:

In the conventional spectrophotometric method using either a single or dual wave length technique, the following steps are essentially involved. First the background absorbance A_o of a sample solution containing a free metal ion concentration of $[M]_o$ in a cell of path length b is measured. A precisely measured amount of indicator I is

then added to give a total of F_I mmoles of indicator per mL of sample solution and a second absorbance A_I is measured. The quantity of the total metal ion complexed by the indicator I is then calculated using the relation.

$$[MI]_1 = A_1 - A_0 - (\Sigma E) b F_1 / b(E_{MI} - \Sigma E) \qquad (1)$$

Where: E_{MI} is the molar absorptivity of MI at the wavelength used

$\Sigma E = f_{H2I} E_{H2I} + f_{HI} E_{HI} + f_I E_I$

E_{H2I}, E_{HI} and E_I are the molar absorptivities for H_{2I}, H_I and I at the wavelength used, and f_{H2I}, f_{HI} and f_I are the fractions of the uncomplexed indicator present as H_{2I}, H_I and I.

The free metal ion in the sample solution can then be calculated by

$$[M]_1 = [MI]_1 / K'_1(F_1 - [MI]_1) \qquad (2)$$

where K'_I is the conditional formation constant ($f_I \times K_I$) for the metal indicator complex; K_I is the thermodynamic formation constant for the metal indicator complex.

Rearrangement of equation (2) gives:

$$[MI]_1/[M]_1 = K'_1(F_1 - [MI]_1) \qquad (3)$$

Equation (3) shows that whenever K'_I and/or F_I becomes large, the ratio of $[MI]_I$ to $[M]_I$ becomes large, and the determinate error in the original free metal ion concentration E_M, defined as $[M]_0 - [M]_I$, may become significant. However, the magnitude of E_M depends also on the

degree to which $[M]_0$ is buffered by the auxiliary ligand L. For example, if $[M]_0$ is unbuffered,

$$[M]_0 = [M]_I + [MI]_I \tag{4}$$

while if highly buffered,

$$[M]_0 \approx [M]_I \tag{5}$$

Because the degree of buffering in an actual sample solution may lie any place between these two extremes, $[M]_0$ may lie any place between the two limits established in equations 4 and 5. Therefore, significant determinate error may result, unless $[M]_I$ is very small.

2.1.3 Quantitative Considerations of Metal Ion Buffering:

In theory, if the degree of metal ion buffering, i.e. the extent to which $[M]_0$ is buffered, is known the values of $[M]_I$ and $[MI]_I$ can be used to obtain an improved estimate of $[M]_0$. A correction of this kind requires knowledge of the extent to which the concentration of M changes as a result of formation of MI, i.e. the degree to which $[M]_0$ is buffered. This relationship can be obtained as follows. For an original solution containing the species M, ML, L, HL, and H_2L in equilibrium with each other, the mass balance relationship for the total metal is the sum of $[M]$ and $[ML]$. After the indicator has been added, the mass balance becomes

$$F_M = [M] + [ML] + [MI] \tag{6}$$

in which F_M is the original total metal concentration in the solution. For convenience, a conditional total metal ion concentration F'_M is defined as

$$F'_M = F_M - [MI] \tag{7}$$

From equations 6 and 7

$$F'_M = [M] + [ML] \quad (8)$$

Combining equation 8 and the formation constant expression for ML,

$$K'_L = [ML] \,/\, [M]\,(F_L - [ML]) \quad (9)$$

where K'_L is the conditional formation constant ($f_L \times K_L$) for the metal auxiliary ligand complex,
K_L is the thermodynamic formation constant for the metal auxiliary ligand complex,
F_L is the total mmoles of auxiliary ligand per mL of the sample solution,
and solving the resulting quadratic equation [M] gives, as the positive root, the relation

$$[M] = \frac{\sqrt{1 + K'_L F_L - (K'_L F'_M)^2 + 4K'_L F^4_M - (1 + K'_L F_M - K'_L F'_M)}}{2K'_L} \quad (10)$$

Equation 10 shows the dependence of [M] upon F'_M at various levels of metal ion buffering (i.e. at various values of K'_L and F_L). The change in [M] resulting from changing F'_M can be obtained by taking the partial derivative of [M] with respect to F'_M at constant K'_L and F_L, $(\partial [M] \,/\, \partial F'_M)\, K'_L, F_L$, where

$$\left(\frac{\partial [M]}{\partial F'_M}\right)_{K'_L, F_L} = 1 + \frac{(1 - K'_L + K'_L F'_M)}{\sqrt{(1 + K'_L + F_L - K'_L F'_M)^2 + 4K'_L F'_M}} \quad (11)$$

Because F_M in equation 7 is constant,

$$\left(\frac{\partial F'_M}{\partial [MI]}\right)_{K'_L, F_L} = -1 \quad (12)$$

and therefore,

$$\left(\frac{\partial[M]}{\partial[MI]}\right)_{K'_L,F_L} = \left(\frac{\partial[M]}{\partial F'_M}\right)_{K'_L,F_L}\left(\frac{\partial F'_M}{\partial[MI]}\right)_{K'_L,F_L}$$

$$\left(\frac{\partial[M]}{\partial[MI]}\right)_{K'_L,F_L} = -\frac{\partial[M]}{\partial F'_M} \qquad (13)$$

Thus,

$$\left(\frac{\partial[M]}{\partial[MI]}\right)_{K'_L,F_L} = 1 + \frac{(1 - K'_L F_L + K'_L F'_L)}{\sqrt{(1 + K'_L F_L - K'_L F'_M)^2 + 4K'_L F'_M}} \qquad (14)$$

Equation 14 defines the extent of buffering of the original metal ion concentration by the auxiliary ligand. The reciprocal of the partial derivative in equation 14, $1/(\partial[M]/\partial[MI])K'L,FL$, is the metal ion buffering capacity of the original sample solution and is conceptually analogous to pH buffer capacity (77). In the extreme, the partial derivatives reduce to:

$$\left(\frac{\partial[M]}{\partial F'_M}\right)_{K'_L,F_L} = 1 \; ; \; \left(\frac{\partial[M]}{\partial[MI]}\right)_{K'_L,F_L} = -1 \qquad (15)$$

for completely unbuffered free metal ion solution, and to

$$\left(\frac{\partial[M]}{\partial F'_M}\right)_{K'_L,F_L} = \frac{1}{1+K'_L F_L} \; ; \; \left(\frac{\partial[M]}{\partial[MI]}\right)_{K'_L,F_L} = -\frac{1}{1+ \quad {}_L F} \qquad (16)$$

for those in which the free metal ion is highly buffered.

Table 1, taken from Fulton's work, shows the value of $(\partial[M]/\partial[MI])K'L,FL$ calculated by computer using equation 11 over a range of values for

F'_M, K'_L and F_L. As predicted by equations 15 and 16, the value approaches unity at low buffering (high F'_M relative to degree of buffering) and $1/(1+K'_L F_L)$ at high buffering (low F'_M relative to degree of buffering). In effect, the three parameters F'_M, K'_L and F_L define a three dimensional space, and the value of the free metal ion concentration represents a surface in this space with the individual points being fixed by the values of F'_M, K'_L and F_L. Figure 2.2. shows (11) a plot of computer calculated values of the relationship between [M] and F'_M. The curves shown indicate only three of many possible levels of metal ion buffering; the slope at any point on a given curve is the value of $(\partial [M]/\partial F'_M)K'L,FL$ for the values of F'_M, K'_L and F_L at that point. Again, in the limits of low and high buffering, the slope indicated on the linear regions of the curve are those predicted by equations 15 and 16.

2.1.4 Quantitative Aspects of the Conventional Spectrophotometric Method:

The concepts presented in the previous section can be used to assess the errors introduced by the conventional spectrophotometric method currently being used. In an unknown sample the buffering parameters, K'_L, F_L, are unknown, as are the values of $[M]_0$ (free metal ion concentration), F_M and therefore F'_M. Designating concentrations pertaining to the original solution with subscript 0, and concentrations in the solution after adding an increment of indicator with subscript 1, the following mass balance relationships can be obtained from equation 7:

Table 2.1.

Computer Calculated Values of $\{\partial[M]/\partial F'_M\}K'_L,F_L$ as a function of F'_M, K'_L and F_L.*

			pF'_M						
K'_L	F_L	K'_LF_L	6	5	4	3	2	1	0
10^3	0	0				1.00			
10^3	10^{-3}	1		0.50	0.52	0.72	0.99	1.00	
10^5	10^{-3}	100			0.01	0.52	1.00		
10^3	10^{-1}	100					0.01	0.52	1.00
10^7	10^{-5}	100	0.01	0.52	1.00				

* From R.B.Fulton, unpublished research, University of Alberta.

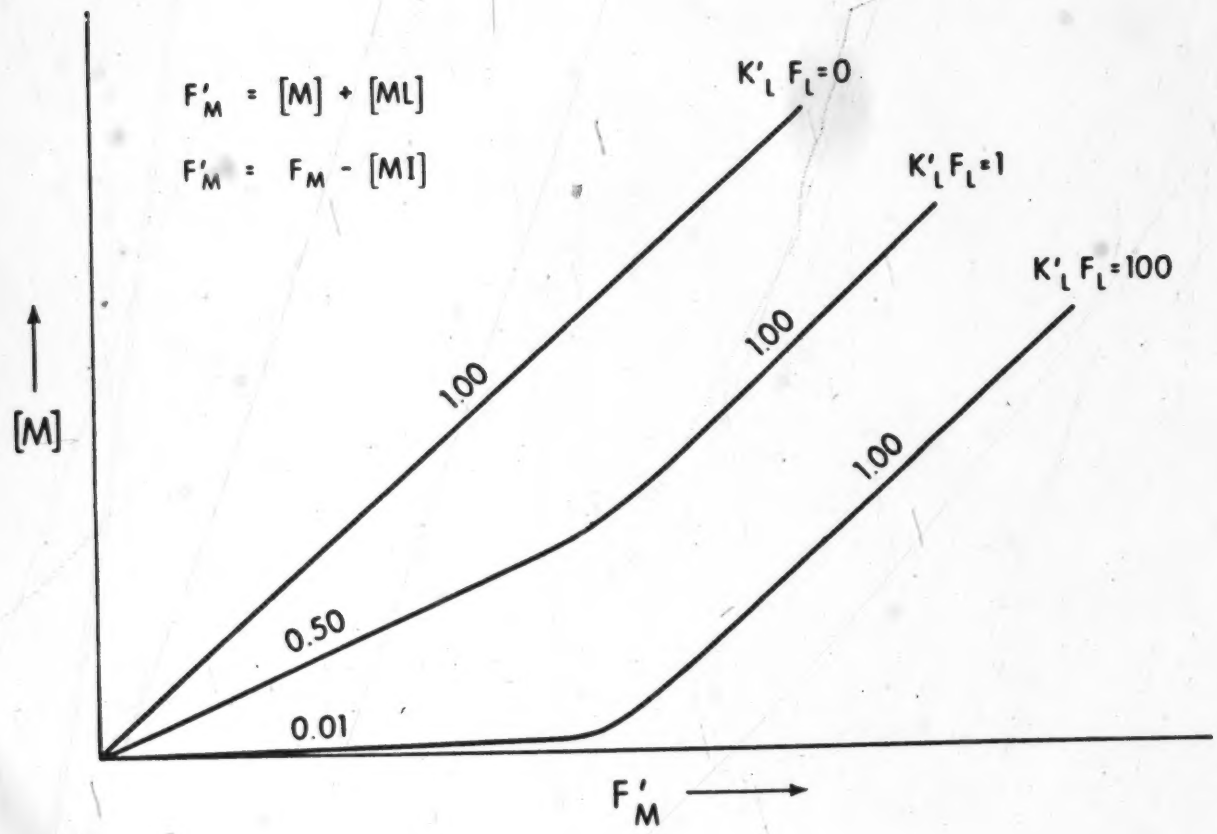


Figure 2.2 Relation between free metal ion concentration [M] and F'_M at various levels of metal ion buffering

$$F'_{M0} = F_M \quad (17)$$

$$F'_{M1} = F_M - [MI]_I \quad (18)$$

$$\Delta F'_{M0-I} = F'_{M0} - F'_{MI} = [MI]_I \quad (19)$$

The concept of the conventional spectrophotometric measurement is depicted in figure 2.3, on an expanded section of a hypothetical curve as shown in figure 2.2. Point 0 refers to the original unknown sample, and point 1 to the sample after addition of the indicator. At point 1, A_I is measured; $[MI]_I$ and, finally, $[M]_I$ are determined using equations 1 and 2. The determinate error in the measured value of $[M]_I$, E_M, is the result of the shift in equilibrium and its magnitude depends on the location of point 0 on the original curve and on the magnitude of $\Delta F'_{M0-I}$ (i.e. $[MI]_I$, see equation 19). In practice a trade-off exists between the determinate error E_M caused by the addition of the indicator and the experimental error associated with the spectrophotometric measurement of the small value of $[MI]_I$. If $[M]_0$ is small and the system is poorly buffered, then to improve the precision of the spectrophotometric measurement $[MI]_I$ must be increased either by using an indicator with a larger K'_I, or by increasing F_I. However, increasing $[MI]_I$ increases $\Delta F'_{M0-I}$, and thus the theoretical error E_M. As mentioned earlier, conditions are generally chosen such that the ratio of $[MI]_I/[M]_0$ is small (0.01 or less), but in solutions where the degree of metal ion buffering is undefined, or poorly defined, meeting these conditions usually is not possible. This results in unfavourable spectrophotometric precision.

A correction for the effect of variation in the metal ion buffering can lead to a powerful technique for the measurement of free metal ion concentration (next section) as evident in figure 2.3. The straight line drawn between points 1 and 0 has a slope, $(\partial[M] / \partial F'_M)_{1-0}$, that can be used to extrapolate from point 1, where experimental measurements

were made, back to point 0, the original sample solution. Mathematically, the relationship is:

$$[M]_0 = [M]_1 + (\partial[M]/\partial F'_M)_{1-0} \times \Delta F'_{M0-1} \qquad (20)$$

Combining equation 20 with rearrangement of equation 19 yields

$$[M]_0 = [M]_1 + (\partial[M]/\partial F'_M)_{1-0} \times [MI]_1 \qquad (21)$$

Because $[MI]_I$ and $[M]_I$ are calculated from the absorbance A_1 by equations 1 and 2, equation 21 could be used to calculate $[M]_0$ if $(\partial [M]/\partial F'_M)_{1-0}$ were known. Unfortunately, in most sample solutions the degree of metal ion buffering, and thererore the value of $(\partial [M]/\partial F'_M)_{1-0}$ is not known. It can, however, be estimated by the procedure described in the next section.

2.1.5. A Modified Spectrophotometric Method: The Sample Ion Increment Method (SIIM)

The sample ion increment method takes advantage of the fact that addition of a known increment of [M] allows more precise estimation of $[M]_0$ in the sample solution. In this approach the value of $(\partial [M]/\partial F'_M)_{1-0}$ is estimated experimentally so that equation 21 can be used to obtain a better estimate of $[M]_0$. The concept of the SIIM is depicted in figure 2.4 on an expanded section of an [M] vs F'_M plot. Points 0 and 1 in figure 2.4 are obtained in exactly the same way as in the single measurement (conventional) technique in figure 2.3. However, after measuring A_1 at point 1, an accurately known increment (ΔF_M) of the metal ion being determined is added (in such a way that dilution effects are negligible) and the absorbance A_2 at point 2 is measured. The concentrations of $[MI]_2$ and $[M]_2$ are calculated as before. The important mass balance

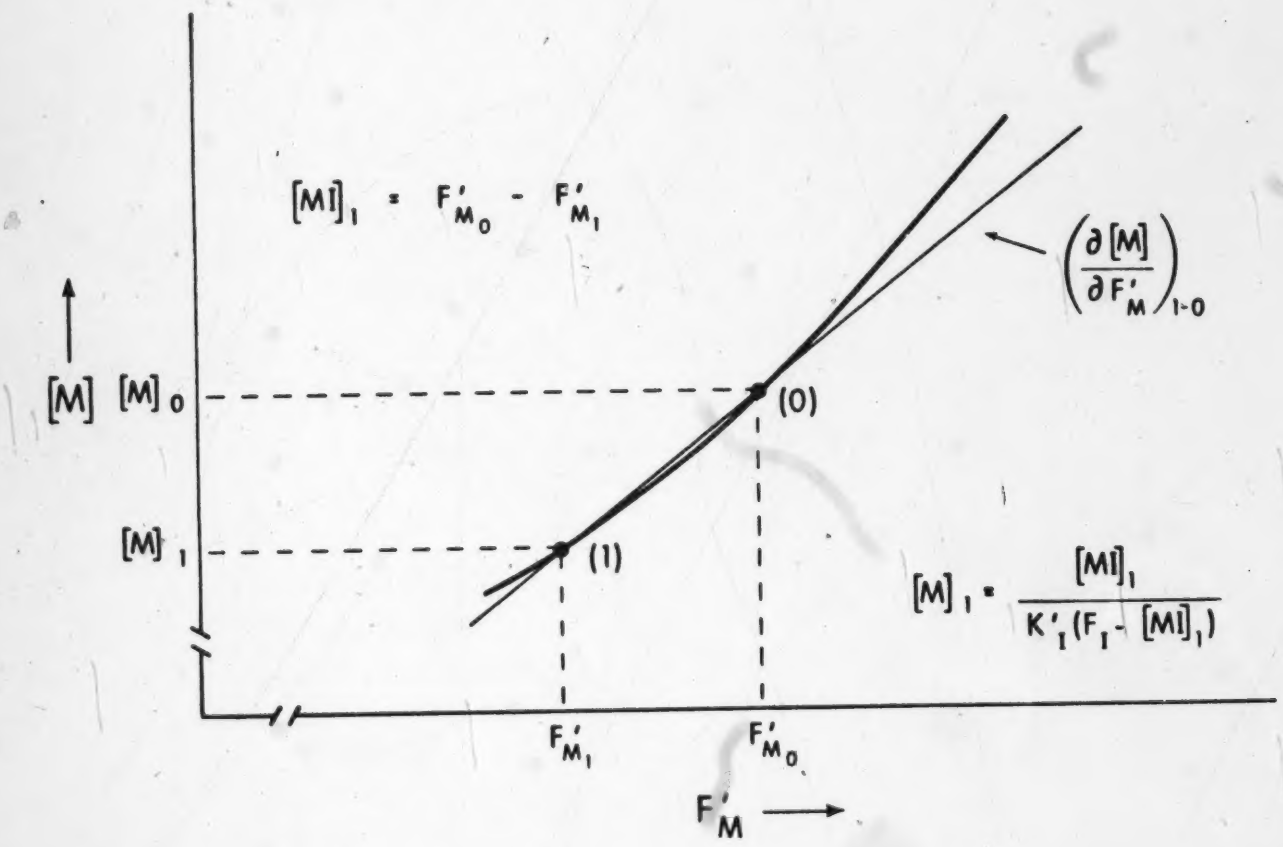


Figure 2.3 Expanded section of a curve from figure 2.2 illustrating the single measurement method

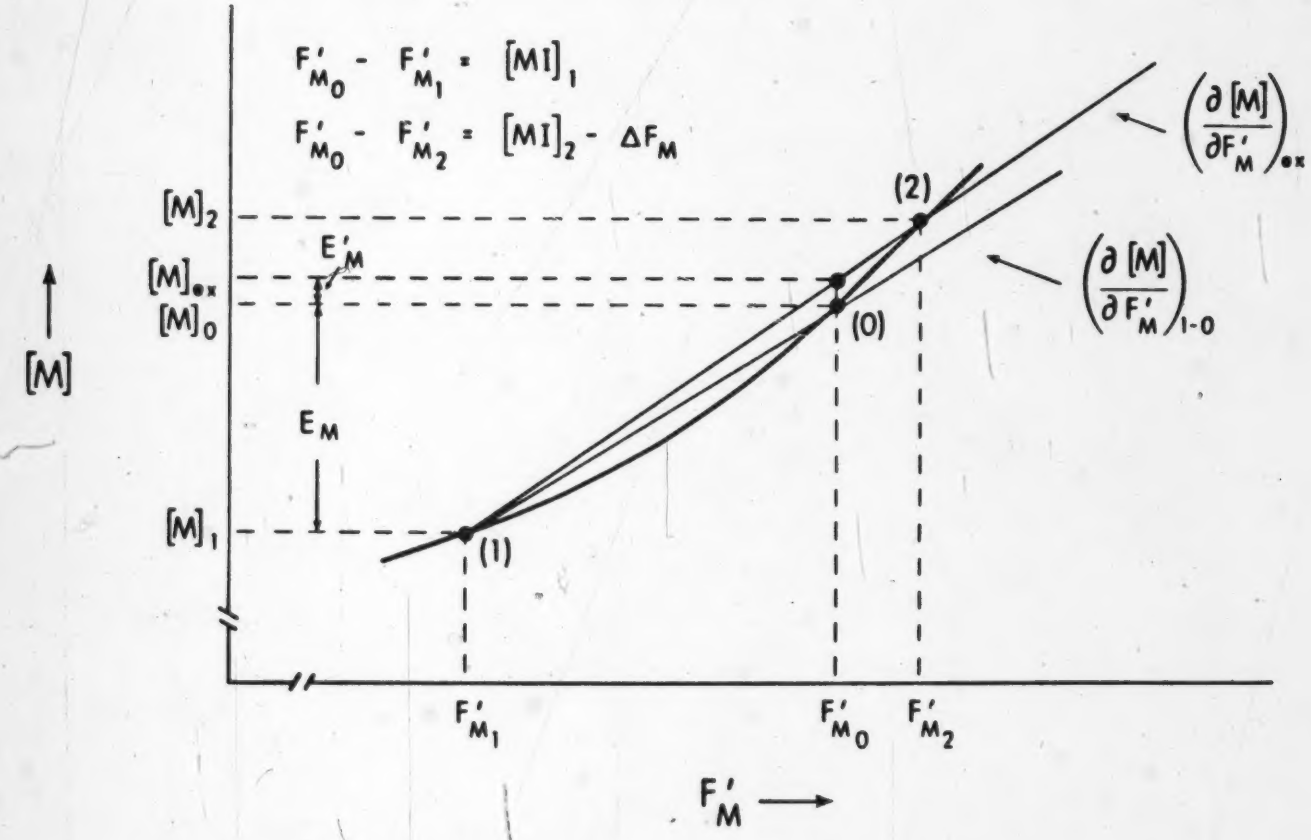


Figure 2.4 Expanded section of a curve from figure 2.2 illustrating the sample ion increment method

relationships in this system are:

$$F'_{M1} = F'_{M0} - [M1]_1 \quad (22)$$

$$F'_{M2} = F'_{M0} + \Delta FM - [M1]_2 \quad (23)$$

$$\Delta F'_{M1-2} = F'_{M1} - F'_{M2} = [M1]_2 - [M1]_1 - \Delta F_M \quad (24)$$

$$\Delta [M]_{1-2} = [M]_1 - [M]_2 \quad (25)$$

An experimental slope $(\partial [M]/\partial F'_M)_{exp}$ can now be determined by the equation,

$$\left(\frac{\partial [M]}{\partial F'_M}\right)_{exp} = \left(\frac{\partial [M]}{\partial F'_M}\right)_{1-2} = \frac{\Delta [M]_{1-2}}{\Delta F'_{M1-2}} = \frac{[M]_1 - [M]_2}{[M1]_2 - [M1]_1 - \Delta F_M} \quad (26)$$

Because $[M]_1$, $[M]_2$, $[M1]_1$; $[M1]_2$ and ΔF_M are either known or experimentally determinable, the value of the experimental slope can now be calculated. The experimentally determined value of the free metal ion $[M]_{exp}$ can then be calculated from the equation,

$$[M]_{exp} = [M]_1 + (\partial [M]/\partial F'_M)_{exp} \times [M1]_1 \quad (27)$$

The close agreement between $[M]_{exp}$ and $[M]_0$, as opposed to the poor agreement between $[M]_1$ and $[M]_0$, is clearly evident from figure 2.4. The determinate error in this E'_M is the difference between $[M]_0$ and $[M]_{exp}$ and results from the inequality of the two slopes $(\partial [M]/\partial F'_M)_{exp}$ and $(\partial [M]/\partial F'_M)_{1-0}$. The relationship is:

$$E'_M = \left(\frac{\partial [M]}{\partial F'_M}\right)_{1-0} - \left(\frac{\partial [M]}{\partial F'_M}\right)_{exp} \times [M1]_1 \quad (28)$$

E'_M depends on: 1) the magnitude of $[MI]_1$, controlled experimentally by K'_I for the indicator chosen and the concentration of the indicator used, F_I ; 2) the magnitude of the two slopes $(\partial [M]/\partial F'_M)_{1-0}$ and $(\partial [M]/\partial F'_M)_{exp}$, determined by the original sample solution, as well as the choice of K'_I, F_I, and ΔF_M ; 3) the difference in the magnitude of the two slopes, determined by the choice of K'_I, F_I and ΔF_M. From figure 2.4 it is seen that if $F'_{M2} = F'_{M0}$, the determinate error of E'_M becomes zero.

For F'_{M2} to equal F'_{M0}, F_M must be equal to $[MI]_2$ (equation 23). But $[MI]_2$ cannot be known until ΔF_M has been added and A_2 measured. Generally, however, $[MI]_2 \simeq [MI]_1$, and E'_M can be minimized by making $\Delta F_M \simeq [MI]_1$. In extreme cases an iterative approach can be used in which several identical samples are analyzed successively, the value of $[MI]_2$ from the previous analysis being used to pick ΔF_M for the next analysis.

2.2 SELECTION OF A METALLOCHROMIC INDICATOR FOR THE DETERMINATION OF IONIC CALCIUM CONCENTRATIONS

The selection of the best indicator for any spectrophotometric method for the determination of metal ion still remains a difficult problem, and the situation for calcium is not any different from other metals. There exists not only a great variety of commercially available metallochromic indicators, but also lists of indicators which have been synthesised in research laboratories for the determination of ionic calcium. A multitude of indicators have been described (78-81), but the information available about their properties is rarely extensive enough to allow an objective selection. At best, the guidelines available comprise a description of their properties, values of acid dissociation constants and the

equilibrium constants with the metal ions which most of the time are of doubtful stoichiometries and especially magnitudes for calcium and magnesium. Therefore when a particular metallochromic indicator is considered for the determination of calcium, a fairly detailed investigation is required before it can be employed for any quantitative measurements.

The literature reveals that spectrophotometric measurement of ionic concentrations of calcium is usually done using murexide, tetramethyl-murexide, antipyrylazo-III and arsenazo-III (78-80). Among them, arsenazo-III seems to be the most frequently used indicator for ionic calcium determinations in biological systems. Thomas (79) has classified these metallochromic indicators for calcium into three different categories according to their structure, affinity towards calcium and selectivity over other ions, especially magnesium. The first category of indicators includes murexide and tetramethyl-murexide. These compounds have a substantially lower affinity for calcium than the others and suffer considerable interference from other ions, especially magnesium and sodium (49). Moreover, the utility of these indicators is further limited by the fact that the absorbance changes on binding to calcium are not very large and occur at a shorter wavelength than is usually desirable. The second category of indicators includes the azo dyes, of which antipyrylazo-III and arsenazo-III are representative members. These dyes have been used extensively in the last decade. The selection of the second category of indicators over the first one is based mainly on a much larger affinity for calcium, higher sensitivity and fewer interferences. However, these indicators have not been fully characterized and there exists controversy about reaction stoichiometries and stability constant values with calcium (79). The third and final category of indicators, presently under development by Tsien and co-workers (62-64,82), are difficult to synthesize and are not commercially available at this time. Reported advantages over the other compounds

are a much higher affinity towards calcium and fewer interferences and hence better selectivity.

2.2.1 The Choice of the Indicator Molecule and the SIIM Requirements for Indicators

The properties of the indicators murexide, tetramethyl-murexide, antipyrylazo-III and arsenazo-III are summarized in table 2.2. Arsenazo-III was selected for study in this work becuase of its higher sensitivity and selectivity, as evident from the data in table 2.2. For calcium-arsenazo-III complexes the literature reveals that the reaction stoichiometries and their formation constants values are in dispute. The results of different workers on calcium-arsenazo-III are tabulated in table 2.3. It is evident from table 2.3 that there is a need for a detailed study to establish the nature of the various species of calcium-arsenazo-III present and their conditional formation constants at pH values near 7.

Arsenazo-III was selected as an indicator ligand for the determination of ionic concentrations of calcium by the SIIM because it met most closely the requirements imposed on the indicator ligand by the technique. Two requirements of the SIIM approach are that the indicator be of known purity, and that the conditional formation constant(s) of the metal-ligand system be known at that pH and the ionic strength of the sample. Moreover the interference from magnesium is less, since measurements are made at 660nm where the magnesium-arsenazo-III absorbance is negligible. However Mg-arsenazo-III does interfere if the measurements are made at 600nm, the first absorption maxima of calcium-arsenazo-III complex (Figure 2.5).

Since the reaction stoichiometries and the conditional formation constants for calcium arsenazo-III have not been fully characterized

Table 2.2

Properties of Metallochromic Indicators for Calcium :

	Murexide	Tetramethyl Murexide	Antipyriazo III	Arsenazo III
Molecular Weight	302	340	746	776
Stability of aqueous solution	not stable	not stable	stable	stable
Predominant stoichiometry	1:1	1:1	N E	N E
Apparent stability constants (pH=7) with calcium	280	297	N E	*
with magnesium	78	109	N E	N E
Suitable pair of wavelength (nm)	500-544	520-550	670-690	660-690
Linear relation to Ca^{2+}	good	good	poor	good
Mg^{2+} interference	yes	yes	yes	no
Na^{+} interference	yes	yes	no	no
Affected by pH change	yes	yes	no	yes

N E not established

* several values reported; see table 2.3

Table 2.3

Stability Constants of Calcium-Arsenazo III Complexes :

Reaction Stoichiometry [Log Stability Constants]				References
1:1	1:2	2:1	2:2	
4.77				144
5.28	0.87			145
4.82	0.98			146
4.35	3.59	1.04	4.07	147
5.8	3.5	2.23		148
4.3				149
5.4				150
4.6				151
3.52				152

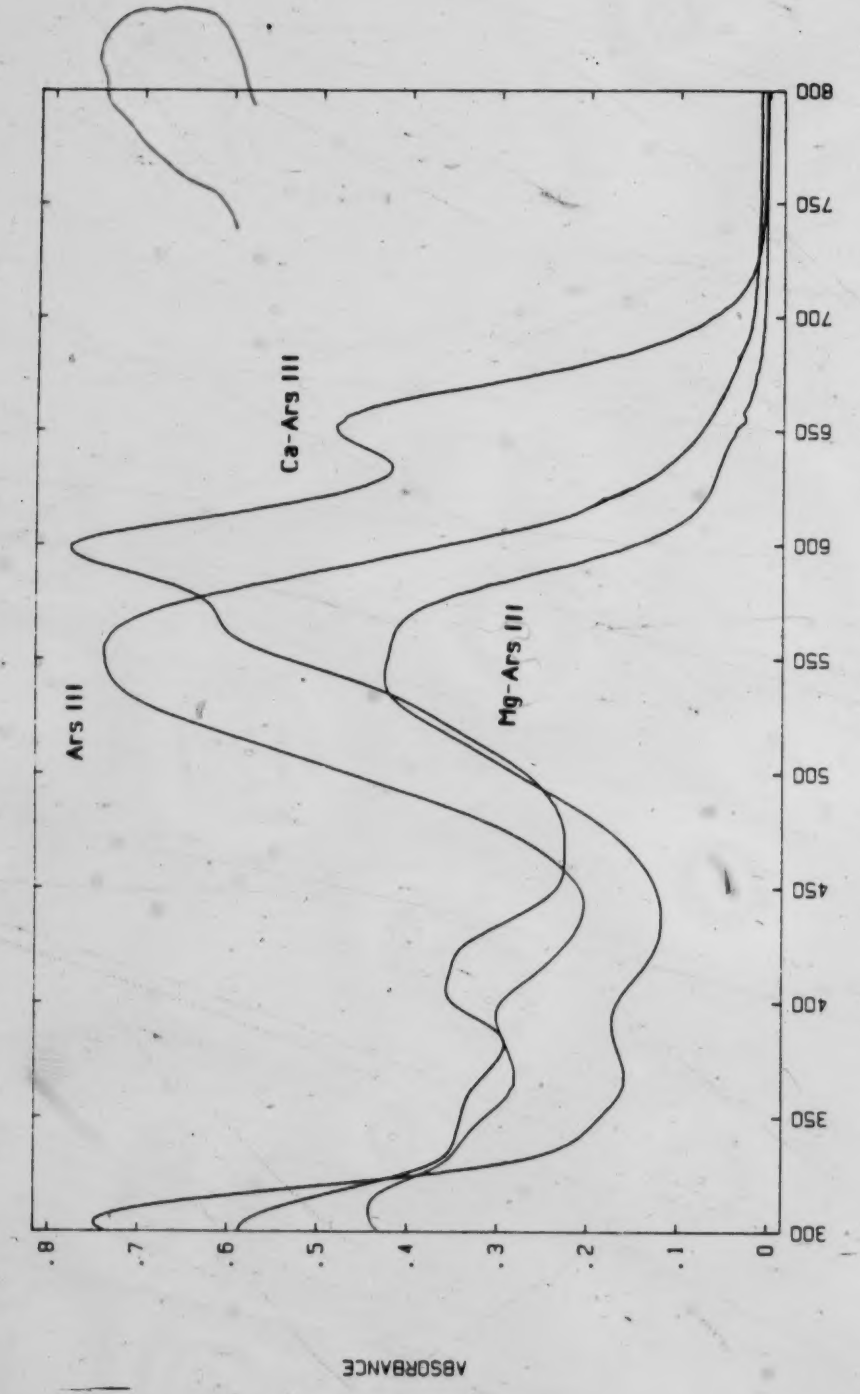


Figure 2.5 Visible absorption spectra of arsenazo-III, calcium arsenazo-III and magnesium arsenazo-III complexes

(table 2.3), and the protonation constants have been reported on material of uncertain purity, a study was undertaken to establish these values and test the system on the measurement of free (ionic) concentrations of calcium by SIIM in biological fluids.

2.3 DETERMINATION OF PROTONATION CONSTANTS OF ARSENAZO-111 AND CONDITIONAL FORMATION CONSTANTS OF THE CALCIUM-ARSENAZO-111 SYSTEM.

2.3.1. Experimental:

Chemicals and Reagents:

Calcium Solution: A stock solution of 5.0×10^{-3} M calcium solution was prepared from calcium nitrate (Anachemia) and enough 1M MOPS (morphine propane sulfonic acid) was added to adjust the pH to 7.25 ± 0.05.

Sodium Hydroxide Solution: Solutions ranging from 3.0×10^{-2} M to 7.0×10^{-3} M NaOH were prepared by dilution of aqueous saturated NaOH (because Na_2CO_3 precipitates in saturated NaOH) solution and standarized against potassium hydrogen phthalate (Fisher). Using phenolphthalin as an indicator the solutions were used within a few hours of preparation to minimize carbon dioxide absorption.

Arsenazo-111 Solution: A 1.0×10^{-4} M arsenazo-111 solution (Sigma) was prepared which was further diluted as required.

Standard buffers of pH 7.00 ± 0.01 (Fisher) and pH 10.00 ± 0.01 (Anachem) were used to calibrate the glass pH electrode. A certified solution of calcium (Fisher) was used for the calibration of the calcium ion selective electrode (Orion Research Inc.).

2.3.2 Apparatus:

Spectrophotometric and potentiometric titrations were performed using a minicomputer (PDP 11/03, Digitial Electronic Corp.) controlled gravimetric titrator which has been described earlier (83). The titrant is placed in a 250-mL propylene bottle on the pan of a top loading balance (Mettler, Model PL 200) and the titrant fed through 1-mm 1.d Tygon tubing to an isolatch solenoid valve (General Valve Corp. N.J.) and then to the titration vessel, a 200-mL container with a Teflon lid having provisions for inserting the electrodes and input and output from the flow cell. , Electrodes from a pH meter (Fisher, Model Accumet 521) and input and output 0.5-mm Tygon tubing from the flow cell of the spectrophotometer (Cary, Model 118) were also immersed in the titration vessel. Absorbance changes in the titration vessel in the course of titration were monitored by circulating the contents via a peristaltic pump (Gilson, Model Miniplus -2) through a quartz flow cell of 1-cm path length and 10-μL volume (Hellma Cells Corp. N.Y). The flow cells were thermostatted at 25C $\pm$ 1C and the titrations were performed in a constant temperature room held at 25C. The B.C.D. outputs from the spectrophotometer, pH meter and the balance were fed to multiplexer interfaced to the PDP 11/03. A control program written in FORTRAN controlled the experiment. With this arrangement simultaneous monitoring of absorbance and pH could be carried out as a function of the weight of titrant added. The program controlled titrant addition by means of the solenoid valve and read and recorded absorbance values at preselected wavelengths of 360, 550, 600, 660 and 700nm after each addition of the titrant. Readouts for simultaneous monitoring of pH/mv and absorbance consisted of weight of titrant delivered, pH/mv and standard deviation of pH/mv readings, absorbance and standard deviation in the absorbance readings, corrected absorbance and the

first and second derivatives of the absorbance readings.

Titrations which were monitored potentiometrically alone (pH titrations) were carried out in a smaller glass container of 75mL volume provided with a Teflon stopper with holes to accomodate the electrodes and the imput from the solenoid valve (gravimetric buret). The pH electrode used was a combination microelectrode (Fisher) and the potentiometric titrations employing an ion selective electrodes were carried out using a calcium ion selective electrode (Orion) with a calomel reference electrode.

Conventional spectrophotometric measurements were done in a 1-cm quartz cell on a Cary 118 spectrophotometer.

2.3. Procedure:

Protonation Constants of Arsenazo-111: For the determination of the protonation constants of arsenazo-111, 12.5mL of 1.0 X 10^{-4}M arsenazo-111 solution were pipetted into a titration vessel, 5.0mL of 1M KNO_3 and 32.5mL of distilled water (after being passed through a mixed bed ion-exchange resin column) added, and the resulting solution weighed to the nearest 0.1mg. A stream of nitrogen pre-saturated with water vapour was passed gently through the solution for 5 minutes before titrations and over the surface of the solution during the titration; the solution was magnetically stirred. This solution was then titrated with a standarized 7.0 X 10^{-3}M solution of NaOH using the gravimetric titrator in the dual spectrophotometric/potentiometric mode, that is, both pH and absorbance readings were recorded during the course of the titration. The spectrophotometric measurements were made at 550, 600 and 650nm, the absorbance maxima of the free arsenazo-111 dye being at 550nm, and the absorbance maxima of the calcium-arsenazo-111 complex being at 600 and 650nm.

The absorbance readings were recorded at the wavelength of the calcium-arsenazo-111 complex so as to ascertain the purity of the arsenazo-111 dye.

2.4 Conditional Formation Constants of Calcium-Arsenazo-111 Complexes:

Data for the conditional stability constants for the calcium-arsenazo-111 complexes were collected by potentiometric pH titrations alone and by simultaneous spectrophotometric/potentiometric titrations employing the calcium ion selective electrode at a constant pH of 7.25 ± 0.05 and ionic strength 0.1 in KNO_3.

Data for the conditional stability constants of calcium-arsenazo-111 by potentiometric titration were collected by the same titration procedure described for the protonation constants of arsenazo-111, except that 250 µL of 5.0×10^{-3} M calcium solution (representing a calcium to arsenazo-111 mole ratio of 1:1) were added to the solution in each titration vessel and the volume of the water decreased correspondingly to keep the total weight of solution for the two sets of determinations identical. The resulting solutions were then titrated with 7.0×10^{-3} M NaOH solution as before.

The dual spectrophotometric/potentiometric titrations employing the calcium ion selective electrode were performed as follows. A 10-mL portion of 1.0×10^{-4} M arsenazo-111 solution and 5.0mL of 1M KNO_3 were taken in a titration vessel and diluted to 50.0mL with enough MOPS to hold the pH of the solution at 7.25 ± 0.05. This solution was then titrated with 1.0×10^{-4} M calcium solution whose pH and ionic strength was the same as the titrant. The titration was carried out until 30.0gms of 1.0×10^{-4} M calcium was added in increments of 0.5gms and the absorbance and the mV readings were recorded after each addition at preselected values. To a second titration vessel

5.0mL of 1.0 X 10^{-4}M calcium solution and 5.0mL of 1M KNO_3 were taken and diluted to 50mL with enough MOPS to adjust the pH to 7.25 $\pm$ 0.05, the concentration of calcium in this final solution being 1.0 X 10^{-5}M. This solution was then titrated against 1.0 X 10^{-4}M arsenazo-111, whose pH and ionic strength was the same as the titrant. The titration was carried out until 15.0gms of 1,0 X 10^{-4}M arsenazo-111 was added in increments of 0.50gms. The absorbance and the mV readings were recorded as before.

2.5 Results and Discussion:

Most published data on the protonation constants of arsenazo-111 and its formation constants with calcium have been reported on material of doubtful purity. It has been pointed out that commercially available arsenazo-111 contains impurities as a side product of systhesis which complex calcium (84), thus making use of the commercially available product impractible. In this study, the constants were measured on dye which is now commercially available (Sigma Chemicals) in 98% purity.

Because of the relatively high cost of the commercially purified compound and the small yield, when purification is carried out on the impure product, the use of a computer controlled gravimetric titrator offers advantages in terms of accuracy, precision and a substantial saving of time and chemicals. This is because titrations can be carried out on small volumes of samples; titrations consisting of 40 to 500 points can be easily generated on a few milligrams of material.

The data collected from the potentiometric and spectrophotometric titrations of arsenazo-111 with NaOH were processed using the computer program MINIQUAD81 (85-86), a modified version of the original

program MINIQUAD (87-92). These calculations were run on the University of Alberta Amdahl 470V/8 mainframe computer in the initial stages of this work. Subsequently the original FORTRAN source code of MINIQUAD (89) was rewritten on an IBM PC microcomputer in FORTRAN using Microsoft FORTRAN complier. Although it took substantially more time to run the program on the microcomputer, it was preferred owing to the cost of running on a mainframe and because of the user friendliness of the microcomputer. This program employs the Gauss-Newton iterative procedure to refine the β values by minimizing the sum of the squares of the residuals of the measured and the calculated quantities of mass balance equations involving protons, metal ions, ligands and β values. MINIQUAD81 calculates β values according to:

$$aA + bH \rightleftharpoons A^a H^h \qquad (29)$$

$$\beta = [A_a H_b] / [A]^a [H]^b \qquad (30)$$

where A and H are the ligand and proton respectively, and a and b are their stoichiometric coefficients. Thus, for example, for arsenazo-111 (H_8A):

$$[HA] / [H][A] = \beta_1 \qquad (32)$$

$$[H_2A] / [H]^2[A] = \beta_2 \qquad (33)$$

$$[H_3A] / [H]^3[A] = \beta_3 \qquad (34)$$

....

....

$$[H_8A] / [H]^8 [A] = \beta_8 \qquad (35)$$

Charges have been omitted for simplicity in all cases.

Equations 35 to 51 are used to convert these constants to conventional acid dissociation constants.

$$[H]\ [H_7A]\ /\ [H_8A] = K_{d1} \quad (36)$$

$$[H]\ [H_6A]\ /\ [H_7A] = K_{d2} \quad (37)$$

$$[H]\ [H_5A]\ /\ [H_6A] = K_{d3} \quad (38)$$

.....

.....

$$[H]\ [A]\ /\ [HA] = K_{d8} \quad (39)$$

and,

$$[H_8A]\ /\ [H_7A]\ [H] = K_{f8} \quad (40)$$

$$[H_7A]\ /\ [H_6A]\ [H] = K_{f7} \quad (41)$$

$$[H_6A]\ /\ [H_5A]\ [H] = K_{f6} \quad (42)$$

.....

.....

$$[HA]\ /\ [H]\ [A] = K_{f1} \quad (43)$$

$$-\log K_d = \log K_f \quad (44)$$

$$\log \beta_1 = \log K_{f1} = -\log K_{d8} \quad (45)$$

$$\log \beta_2 = \log K_{f1} + \log K_{f2} = -\log K_{d8} - \log K_{d7} \quad (46)$$

$$\log \beta_3 = \log K_{f1} + \log K_{f2} + \log K_{f3} = - \log K_{d8} - \log K_{d7} - \log K_{d6} \quad (47)$$

.....
.....

$$\log \beta_7 = \log K_{f1} + \log K_{f2} + ... + \log K_{f8} = - \log K_{d8} - \log K_{d7} - ..... - \log K_{d1} \quad (48)$$

where β represents the overall formation constants, K_f the stepwise formation constant and K_d the stepwise dissociation constant. From the above, it is quite evident that the ligand proton association constants can be simply converted from MINIQUAD81 β values to conventional pKa values by equations 49 to 52.

$$pKa_1 = \log \beta_8 - \log \beta_7 \quad (49)$$

$$pKa_2 = \log \beta_7 - \log \beta_6$$

$$pKa_3 = \log \beta_6 - \log \beta_5$$

.....
.....

$$pKa_8 = \log \beta_1 \quad (52)$$

The weighted means and the standard deviations of pKa values determined from the treatment of data from several sets of titrations were calculated using the equations:

$$X_M = \Sigma (X/S^2) \; / \Sigma (1/S^2) \quad (53)$$

$$S_W = 1/\sqrt{\left\{\Sigma S^2 \; / \; (n-1)\right\}} \quad (54)$$

where X_M and S_W are the weighted mean and weighted standard deviation, X represents each individual result, S is the standard deviation and n is the number of results used in the calibration. The Q test is used to determine if outlying pKa values should be included in the result (93).

$$Q = \frac{\text{Suspect Value - Nearest Value}}{\text{Largest Value - Smallest Value}}$$

(Compare at 90% confidence level)

From the titration results, the first two protons of the free acid, corresponding to H_8A and H_7A^-, are strongly acidic and appear completely dissociated over the pH range studied here. Also, dissociation of the species H_2A^{6-} and HA^{7-} is too slight to be observed in aqueous solution. Therefore constants corresponding to the following equilibria could be evaluated for arsenazo-III:

$$H_6A^{2-} \rightleftharpoons H_5A^{3-} \rightleftharpoons H_4A^{4-} \rightleftharpoons H_3A^{5-} \rightleftharpoons H_2A^{6-}$$

The procedure followed for evaluating the equilibria among the remaining species essentially consists of setting up the equilibrium model for the system along with its stoichiometric components (ligands, metal and protons) and inputting the estimates of the formation constants of the species proposed in the model which has been described in the appendix. These are used by the program to set up mass balance equations for each element. MINIQUAD81 generates a simulated titration curve, based on the experimental data. This includes volume, weight and concentrations of titrant and solution to be titrated, pH readings (or mv readings if titration was followed by ion selective electrode as in the dual spectrophotometric/ potentiometric titrations) and initial estimates of the formation constants of the porposed species.

This stimulated titration curve is then compared point by point with that obtained experimentally under the specified conditions of temperature, pH and ionic strangth and, on the basis of the results

of the comparison, the formation constants are refined by means of a non-linear least square alogorthium due to Gauss-Newton. This process is repeated until an arbitrary criterion of fitness is achieved or a maximum number of iterations are preformed, which ever happens to occur first. The criterion of fitness is defined by $R_{factor} \ll R_{limited}$ (89,91-92), Here

$$R_{factor} = \frac{\sum_{i=1}^{i=n} W_i (0_i \text{ calc} - 0_i \text{ obs})^2}{\sum_{i=1}^{i=n} W_i (0_i \text{ obs})^2} \quad (55)$$

$$R_{lim} = \frac{\sum_{i=1}^{i=n} W_i E_i^2}{\sum_{i=1}^{i=n} W_i (0_i \text{ obs})^2} \quad (56)$$

In these equations E_i is the residual in the i^{th} equation calculated from estimates of the error in all the experimental quantities using error propagation rules, 0_i calc and 0_i obs are the calculated and the experimentally available values of ex, pH, concentrations etc. and W_i is the weighting factor. A satisfactory fit is assumed if condition $R_{factor} < R_{limited}$ is achieved. ($W_i = \frac{1}{\sigma_{r_i}^2}$, reciprocal of the variance of the residual).

Relative errors in the final constants are calculated with several different estimates of the overall fit of the experimental data.

One of them is the condition that $R_{factor} < R_{limit}$, as discussed earlier. Another critical condition which has to be satisfied in order to achieve the best possible fit is to minimize the sum of the squares of the residuals, which is the sum of the squares of the differences between the experimental data points and those calculated using the resulting constants. The sum of the squares of the residuals, U, to be minimized is given by the equation:

$$U = \sum_{i=1}^{i=n} (0i\,calc - 0i\,obs)2 = \sum_{i=1}^{i=n} (\Delta 0i)2 \qquad (57)$$

In other words, U gives a measure of fit of the titration curve calculated using the program MINIQUAD81 constants to that obtained experimentally. It was observed in this study that the calculated titration curve will resemble closely the experimental titration curve if the value of sum of the squares of the residuals U is less than 10^{-6} and if the condition $R_{factor} < R_{limit}$ is fulfilled.

The algorithm in the program has the capacity to identify the occurrence of unsuccessful iterations for the calculation of the conditional formation constants which lead to divergence instead of convergence to a single value and will reject those particular species from the model, while the constants for the remaining species are further refined. It was observed in this study that if the concentration of a particular species was found to be less than 3% that species is rejected from the model by MINIQUAB81; hence, the better the initial estimate of the various species present the better the results obtained with MINIQUAD81. The initial estimate of the various species present in the solution was determined by the use of the computer program COMIX (102) on the same experimental data obtained from the titration. Thus the initial estimate obtained from the program COMIX was later used as guide for modelling the

species in MINIQUAD81.

The output of MINIQUAD81 provides, in addition to values for the conditional formation constants and an R_{factor}, standard deviations of the constants, a matrix of correlation coefficients between logarthims of the constants, a species distribution plot, a plot of the residuals and a plot of experimental and calculated titration points based on the refined β values.

The results obtained on processing the potentiometric and spectrophotometric/potentiometric titration data using MINIQUAD81 are tabulated in table 2.4. According to the criterion adopted, all models given in the table are satisfactory. Results indicate a larger uncertainty in the values of log K_5 and log K_6 than for others, but these correspond to H_6L^{2-} and H_5L^{3-}, minor species at pH 7. Their existence in solution is better substantiated in the titrations of more concentrated solutions. Problems associated with definition of minor species in data fitting have been discussed (94) and were pointed out earlier. Not withstanding the low concentrations of H_6L^{2-} and H_5L^{3-} observed, the results offer strong evidence for their existence. The values obtained for the formation constants for arsenazo-III in table 2.4 by MINIQUAD81 calculation show excellent agreement with those reported in the literature (95). Figure 2.6. shows experimental data points and a calculated (simulated) titration curve based on the average protonation constants of all the runs obtained by MINIQUAD81 in this study. The fit of the calculated β values to the individual data set is excellent as evident from figure 2.6. A similar good fit was also obtained for the more concentrated set of arsenazo-III solutions.

Table 2.4

Protonation Constants of Arsenazo-III :

Arsenazo-III Concn., (M)	H_3L^{5-} log K3	H_4L^{4-} log K4	H_5L^{3-} log K5	H_6L^{2-} log K6	Kw	R_{factor}
2.00 E-5	8.90±0.03	7.04±0.03	4.78±0.06	3.73±0.06	1.28 E-14	2 E-3
2.60 E-5	8.82±0.07	7.1 ±0.1	4.9 ±0.1	3.5 ±0.1	1.47 E-14	4 E-3
2.60 E-5	9.02±0.02	6.90±0.02	4.51±0.03	2.9 ±0.3	1.31 E-14	2 E-3
6.76 E-4	9.27±0.02	7.19±0.02	4.24±0.02	3.01±0.01	1.34 E-14	2 E-3
6.76 E-4	9.24±0.03	7.19±0.04	4.34±0.04	2.86±0.04	1.38 E-14	3 E-3
6.76 E-4	9.21±0.07	7.15±0.08	4.4 ±0.1	2.88±0.04	1.89 E-14	7 E-3
Average :	9.1 ±0.2	7.1 ±0.1	4.5 ±0.3	3.2 ±0.5		
Literature (95)	9.0	7.1	5.0	3.6		

Titration conditions : For samples 1 to 3 , sample volume = 100 mL , NaoH titrant = 7.5×10^{-3} M ; for samples 4 to 6 sample volume = 12.10 mL , NaOH titrant 3.41×10^{-3} M ; ionic strength adjusted to 0.10 with KNO_3 .

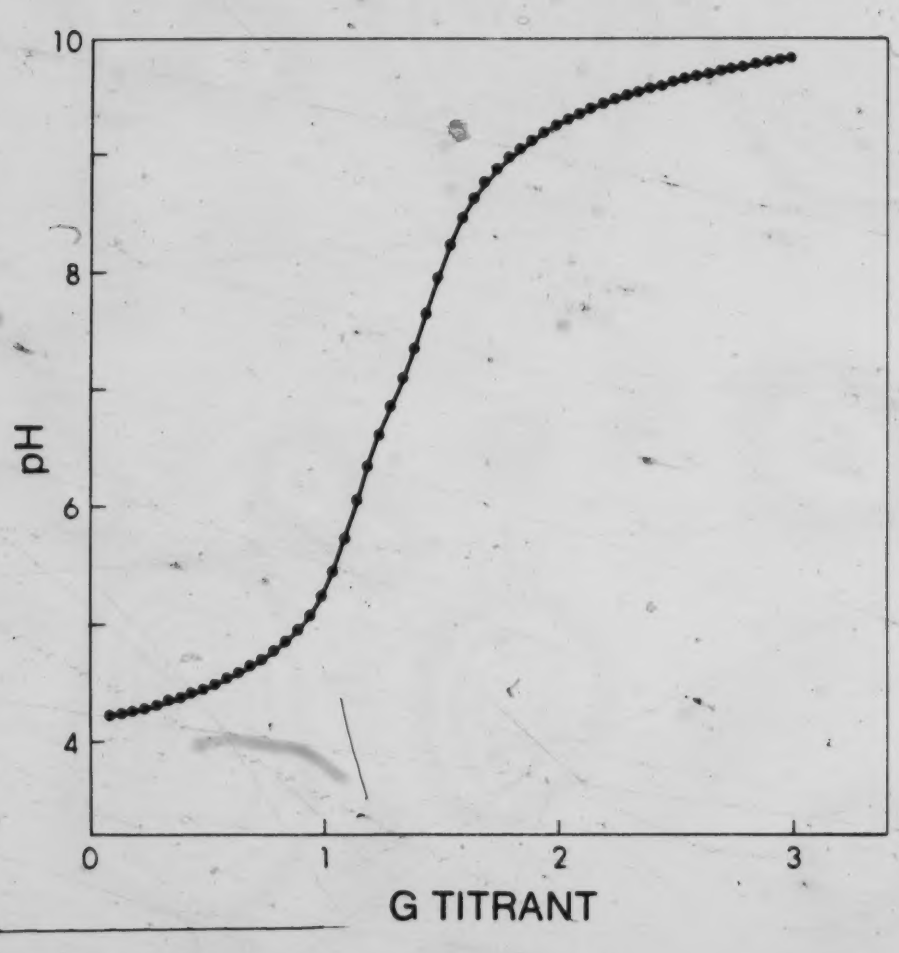
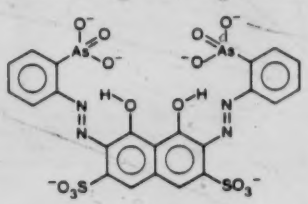


Figure 2.6 pH titration of 2.5 X 10^{-5}M arsenazo-111 in 0.1M KNO_3 with 7.50 X 10^{-3}M NaOH. Experimental points indicated by •, solid line generated by computer using Miniquad81 calculated protonation constants.

2.6 Effect of Proton Dissociation on the Spectrum of Arsenazo-III

The structure of arsenazo-III may be represented as follows:



H_2L^{6-}

As assignment of the dissociating protons can be made based on the spectral behaviour of arsenazo-III during pH titration and the known characteristic acidities of the arsono and sulfono groups. Sulfonic acids are known to be strong and so the first two strongly acidic protons can be assigned to the two SO_3H groups (96). No spectral or potentiometric evidence indicating association of these two protons was observed in this study. The next four dissociating protons would be expected to originate from the arsono groups. Similarity of the protonation constants of benzenearsonic acid [log K for $(HL/H \cdot L)$ = 8.25; and $(H_2L / HL \cdot H)$ = 3.4 at ionic strentgh of 0.10, and 25C] (97-100) and those obtained is this study (table 2.4) along with the spectrophotometric data, support this assignment

A series of visible absorption spectra of arsenazo-III obtained at various points during a pH titration is shown in Figure 2.7. The absorbance maximum for the protonated forms (H_4L^{4-} to H_2L^{6-}) occurs at 540 nm while that of the HL^{6-} form occurs at 578nm. Removal of the first two arsono protons is not accompanied by any spectral shift, whereas removal of the third and fourth arsono protons each causes a bathochromic spectral shift. The spectra indicate an isobestic point that shifts from 585 to 562 nm as the pH is increased from 4.2 to 8.0. This isobestic point may indicate equilibrium between the monoprotonated and diprotonated forms of the first arsono group, while over pH 8.2 to 9.9 the isobestic

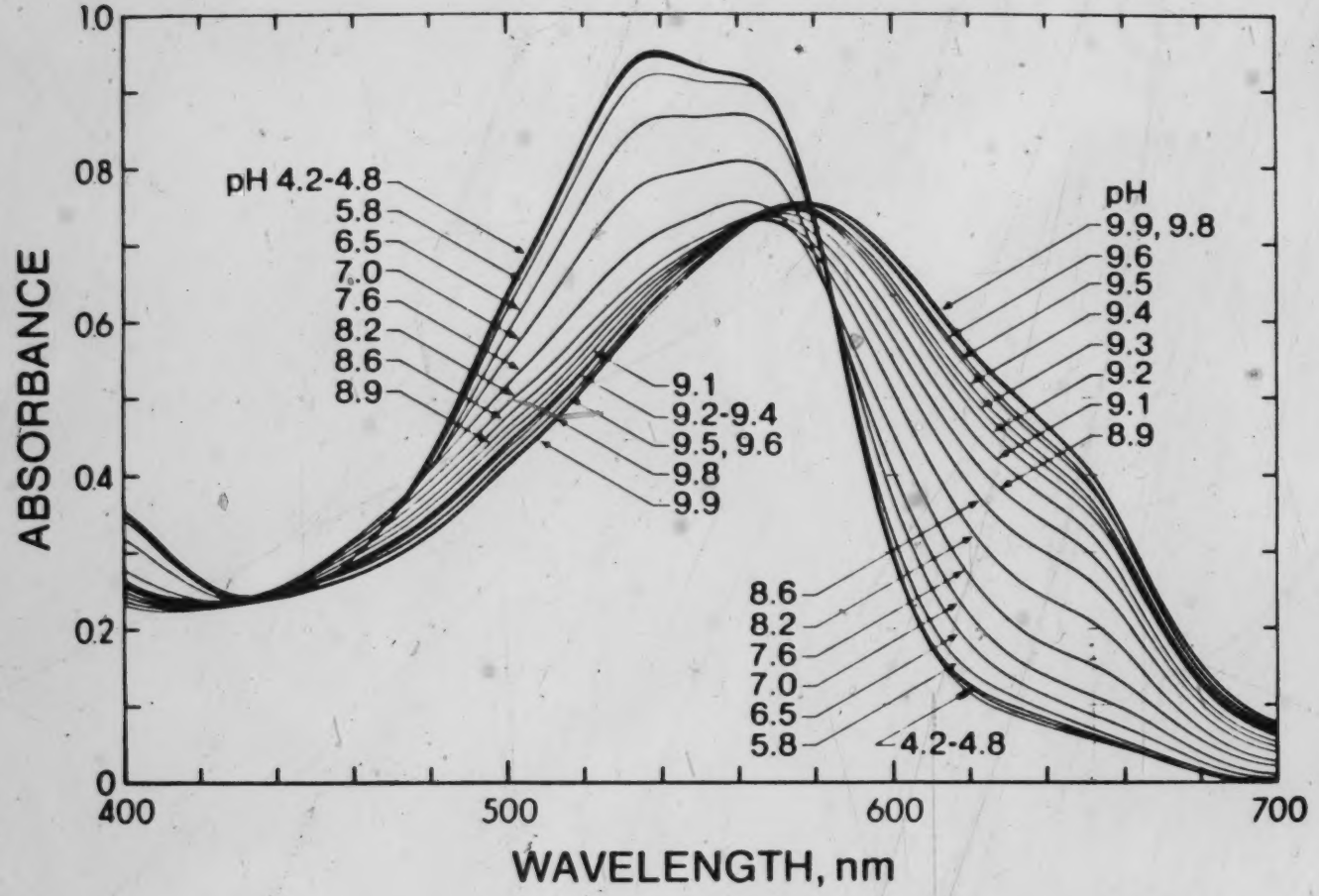


Figure 2.7 Visible absorption spectrum of 7.6×10^{-5} M Arsenazo-III in 0.1M KNO_3 as a function of pH

point may indicate the same equilibra involving the second arsono group. Jaffe (101) has observed that the presence of an arsono group generally does not greatly affect the spectra of the parent aromatic compounds. The electron withdrawing properties of the arsono group do not seem to effect the azo group significantly because no major change in the spectrum is observed upon removal of the first proton from the arsono group.

The dissociation of the sixth proton is clearly observed in the potentiometric titrations, but is not accompanied by any spectral change. The proton is likely removed from the second arsono group, rather than from one of the hydroxyl groups.

Except for the early development of a broad shoulder at 650nm, no further spectral shifts are observed in the titrations at pH values above 9.1, nor does MINIQUAD81 analysis of the potentiometric data indicate further dissociation. It appears, therefore, that the hydroxylic protons undergo little or no dissociation at the concentrations of sodium hydroxide and arsenazo-III studied here.

The various species of arsenazo-III present in aqueous solution in the pH range 4.0 to 12.00 were calculated, using the computer program COMIX (102). A plot of the fraction of various species of arsenazo -III as a function of pH is shown in Figure 2.8.

2.7 Effect of Calcium on the Spectrum of Arsenazo-III and Determination of the Conditional Formation Constants of the System

Figure 2.9 shows absorbance spectra taken at selected points in a pH titration of arsenazo-III as before, but with calcium present at twice the concentration of arsenazo-III. At pH 4.2. the spectrum is characteristic of arsenazo-III alone except for a small peak at

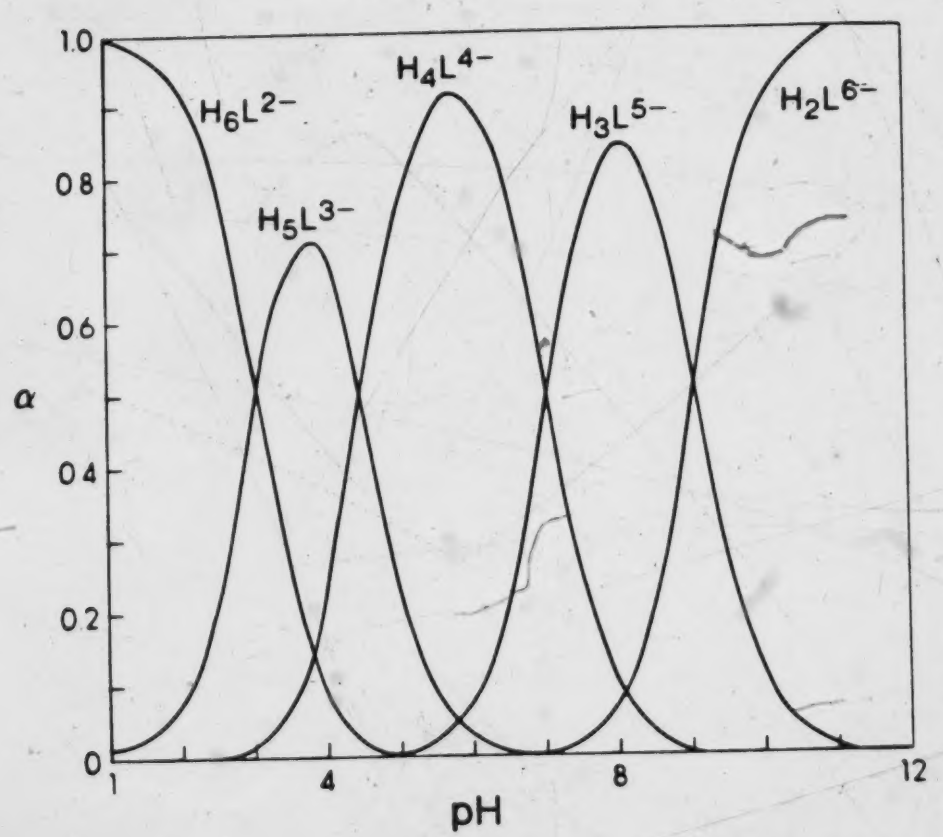


Figure 2.8 Fraction of arsenazo-III present in various ionic forms as a function of pH, calculated by COMIX

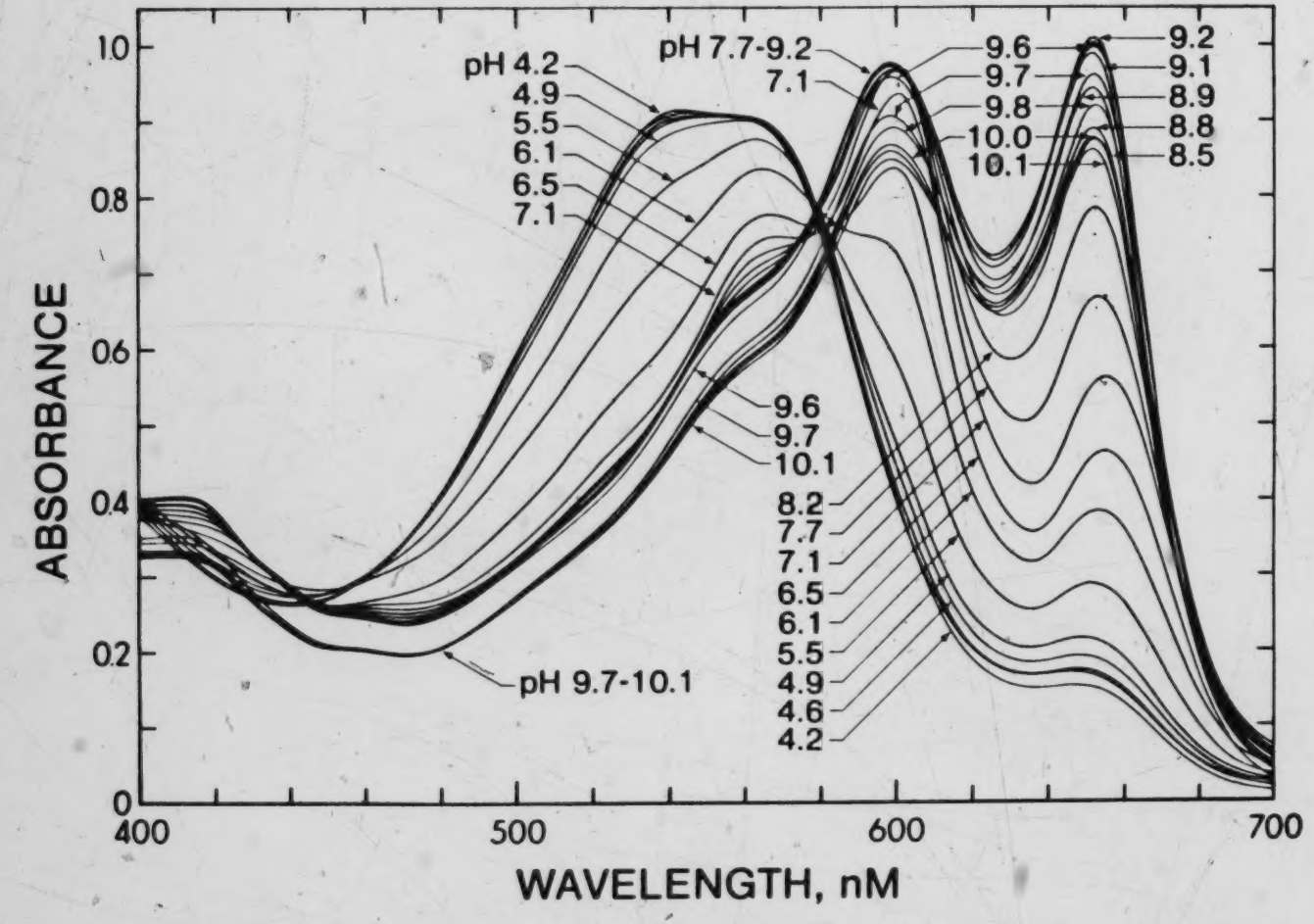


Figure 2.9 Visible absorption spectrum of 2:1 calcium-arsenazo-111 solution in 0.1M KNO_3 as a function of pH. Concentration of arsenazo-111 $7.6 \times \bar{1}0^{-5}\underline{M}$

650 which is due to the absorbance of the calcium complex. Addition of base produces two distinct changes in the specturm. Starting at pH 5.5, the broad maximum at 540-568 nm shifts to 598nm. This change corresponds to the first end point in the pH titration. The second end point in the titration at pH 7.58 corresponds only to a further increase in the absorbance with no spectral shift. Above pH 9.2, absorbance values decrease, perhaps owing to loss of an additional proton from the protonated calcium-arsenazo-111 complex.

Titration plots (weight of NaOH titrant against pH) of arsenazo-111 alone and with calcium are almost superimposible, showing that the presence of calcium does not appreciably affect the acidity of arsenazo-111. In the MINIQUAD81 calculations for the conditional formation constant of the calcium-arsenazo-111 system six dissociable protons were taken into account based on the above studies. The following equilibrium equations were incorporated into the model for MINIQUAD81 data processing:

$$H_2L^{6-} + Ca \rightleftharpoons CaH_2L^{4-}$$

$$H_3L^{5-} + Ca \rightleftharpoons CaH_3L^{3-}$$

$$H_4L^{4-} + Ca \rightleftharpoons CaH_4L^{2-}$$

Table 2.5 summarizes the results of these studies. A model involving the formation of CaH_2L^{4-}, CaH_3L^{3-} and CaH_4L^{2-} was found to give the best fit out of many models considered. The selection of these species were also supported by the results obtained from the computer program COMIX. Although the extent to which any particular species is present in the solution will be governed by the pH and ionic strength of the medium in which the measurement is carried out, at a pH of 7.0 to 7.3 the predominate species are predicted to be CaH_2L^{4-} at pH 7.0 and CaH_2L^{4-} and CaH_3L^{3-} at pH 7.3. This is the pH

Table 2.5

Conditional Formation Constants For Ca-Arsenazo III Complexes :

CaH_2L (log ß1)	CaH_3L (log ß2)	CaH_4L (log ß3)	Kw	R_{factor}
16.41±0.03	8.54±0.05	4.37±0.06	9.43×10^{-14}	7.1×10^{-3}
16.53±0.01	8.63±0.1	4.33±0.05	9.78×10^{-14}	6.6×10^{-3}
16.66±0.04	8.7 ±0.2	4.28±0.01	9.84×10^{-14}	5.8×10^{-3}
16.9 ±0.1	8.8 ±0.2	4.21±0.01	9.7×10^{-14}	6.8×10^{-3}
16.8 ±0.1	8.8 ±0.2	4.22±0.01	9.91×10^{-14}	5.2×10^{-3}
16.7 ±0.1	8.76±0.2	4.25±0.02	9.87×10^{-14}	6.9×10^{-3}

Average Values :

log CaH_2L 16.66

log CaH_3L 8.7

log CaH_4L 4.27

region where most physiological measurements are carried out.

Over the pH range 4.0 to 10.0 the absorption maxima at 650 nm due to the calcium complexes remains constant, but on raising the pH above 12.0 it disappears, as shown in Figure 2.10. The formation of a CaH_2L^{4-} species initially in which calcium is bound to the azo and arsono group on one side of the molecule of arsenazo-III can be postulated. The two maxima might be related to the presence of two weekly interacting chromophoric systems in one molecule of the reagent. Similar phenomena, i.e., the existence of two absorption bands in the visible region, have also been observed in certain complex cyanine dyes having two discrete chromophore systems (104). In the case of an uncomplexed molecule of arsenazo-III, which is symmetrical, both chromophore groups contain identical functional analytical groups. Thus the absorption spectra coincide and there is only one maximum. Complex formation with calcium ion via one group destroys the symmetry of the molecule, and if the system is not one whole with respect to its optical properties, then two absorption maxima develop. Thus only one of the two groups capable of complexing with calcium in the reagent molecule react to form a complex with calcium ion up to pH 10.0, and the predominate species are CaH_2L^{4-} and CaH_3L^{3-}. On raising the pH above 12.0, CaH_2L^{4-} may lose the two hydroxyl protons to form a species in which calcium is coordinated only to hydroxyl oxygens. Further support for these structures is provided by the work of Savvin and Nemodruk (103-104) and a study of the copper arsenazo-III system (105).

2.8 Determination of Free Calcium Concentrations in Synthetic Samples by SIIM:

To investigate the sample ion increment method (SIIM), various synthetic samples were prepared in which the calcium ion buffering was varied over a range by varying the concentration of the auxiliary

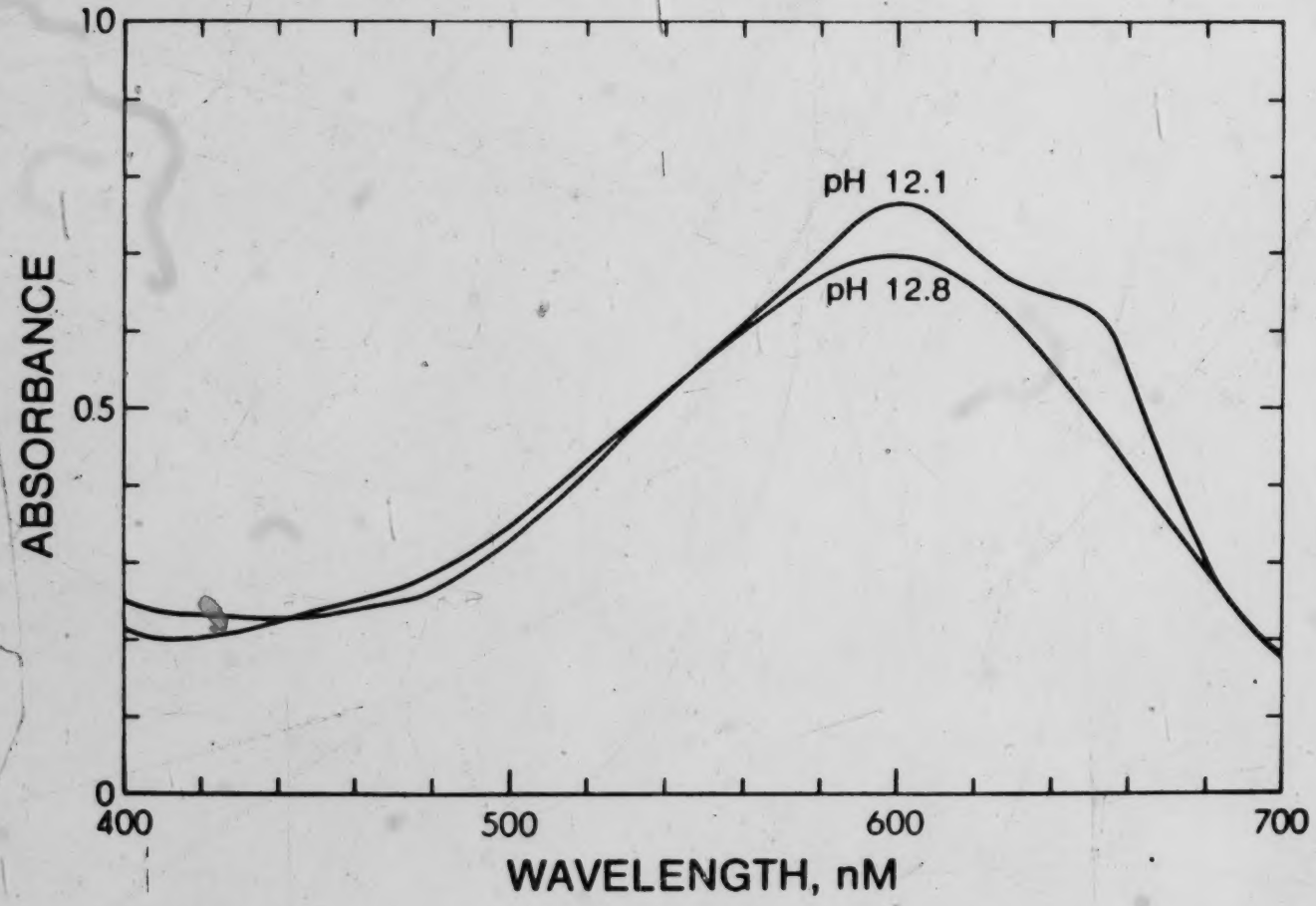


Figure 2.10 Visible absorption spectrum of 2.55×10^{-5} M calcium arsenazo-III in 0.1M KNO_3 at pH 12.1

ligands, thereby achieving differing free calcium concentrations. The auxiliary ligands chosen for this purpose were nitrilotriacetic acid (NTA), phosphate, citrate and tartrate. The conditional formation constant of calcium-auxiliary ligand in each case was calculated using the general equation:

$$K'_{cond} = K_{therm} \times f_L \times f_{Ca}$$

in which K'_{cond} is the conditional formation constant of the calcium - auxiliary ligand complex, K_{therm} is the thermodynamic formation constant and f_L and f_{Ca} are the fractions of the auxiliary ligand and calcium present as the unprotonated ligand anion and the uncomplexed metal, respectively. The value of the conditional formation constant so calculated was used to obtain the free concentration of calcium ion in each case.

2.8.1 Preparation of Synthetic Samples and Measurement of the Free Concentration of Calcium by SIIM

Series A: In these samples the concentration of total calcium was held at 1.0×10^{-5}M and by the addition of varying increments of 0.001M auxiliary ligand NTA, the free concentration of calcium was varied. The pH in each case was maintained at 7.3 ± 0.05 with MOPS buffer and the ionic strength was adjusted to 0.20 with KNO_3. The ionic strength in the final solution was calculated using the Davies equation (49).

Series B: These samples also contained a constant concentration of calcium as in series A (1.0×10^{-5}M) but the auxiliary ligand used to buffer the calcium was citrate. A 0.05M solution of citrate was added in varying increments to vary the free calcium concentration. The pH and the ionic strength were as in series A.

Series C: Conditions were as in series A, but the auxiliary ligand used was 0.05M solution tartrate, which was added in varying increments to a constant total concentration of calcium (1.0×10^{-5}M), in order to vary the free concentration of calcium. The pH and the ionic strength were as in series A.

The scheme for preparing these synthetic samples is summarized in table 2.6. The measurement of the free calcium concentration in these series of solutions essentially involved the following two steps: addition of arsenazo-III indicator and measurement of the absorbance followed by the addition of an increment of calcium and remeasurement of the absorbance of the same solutions. From these two absorbance readings, along with the value of the conditional formation constant of calcium-arsenazo-III and the total concentration of the indicator arsenazo-III added (C_{ind}), the concentration of the free calcium was calculated using the computer program CFMC (calculation of free metal concentration) written in BASIC for an IBM PC microcomputer. This program was based on the equations which were discussed earlier in chapter 2 on the SIIM.

The results of these experiments are tabulated in tables 2.7 to 2.10. It is clear from the values obtained that the results of the SIIM experiments are in excellent agreement with those obtained by calculation.

2.9 Preliminary Measurement of Free Calcium Concentrations in Urine Samples by SIIM

The urine samples used in these studies were provided by the University of Alberta Hospitals. The samples were centrifuged immediately and analysed on the same day. Prior to the analysis of the samples for free calcium by SIIM, a visible spectrum of

Table 2.6

Summary of the Scheme for the Preparation of Synthetic Samples :

C_{ca}	1.00×10^{-5} M in 25 mL of solution
ΔC_{ca}	10 µL of 0.01 M calcium
C_{ind}	2.00×10^{-5} M in 25 mL of solution

Sample	C_{NTA} *, M	$C_{CITRATE}$ *, M	$C_{TARTRATE}$ *, M
1	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-3}
2	1.0×10^{-4}	5.0×10^{-4}	7.5×10^{-3}
3	8.0×10^{-5}	7.5×10^{-4}	1.75×10^{-2}
4	5.0×10^{-5}	1.0×10^{-3}	2.5×10^{-2}
5	2.4×10^{-5}		
6	4.0×10^{-7}		

* Concentrations in 25.00 mL of solution .

Table 2.7

Free Calcium Determination by SIIM in Synthetic Sample :

Sample #	pCa calc	pCa exp	Δ pCa exp-calc
1	5.05	5.01	- 0.04
2	5.17	5.11	- 0.06
3	5.33	5.25	- 0.08
4	5.41	5.36	- 0.05

The synthetic sample contained varying amounts of Tartrate as an auxiliary ligand to achieve varying degrees of calcium ion buffering in solution.

Table 2.8

Free Calcium Determination by SIIM in Synthetic Sample :

Sample #	pCa calc	pCa exp	Δ pCa exp-calc
1	5.01	4.97	- 0.05
2	5.16	5.11	- 0.05
3	5.26	5.20	- 0.06
4	5.32	5.27	- 0.05

The synthetic sample contained varying amounts of Phosphate as an auxiliary ligand to achieve varying degrees of calcium ion buffering in solution .

Table 2.9

Free Calcium Determination by SIIM in Synthetic Sample :

Sample #	PCa calc	PCa exp	Δ PCa exp-calc
1	5.03	4.99	- 0.04
2	5.20	5.23	+ 0.03
3	5.44	5.40	- 0.04
4	5.60	5.58	- 0.02
5	6.0	5.92	- 0.08

The synthetic sample contained varying amounts of NTA as an auxiliary ligand to achieve varying degrees of calcium ion buffering in solution .

Table 2.10

Free Calcium Determination by SIIM in Synthetic Sample :

Sample #	pCa calc	pCa exp	Δ pCa exp-calc
1	5.02	4.98	- 0.04
2	5.4	5.33	- 0.07
3	5.53	5.45	- 0.08
4	5.61	5.54	- 0.07

The synthetic sample contained varying amounts of Citrate as an auxiliary ligand to achieve varying degrees of calcium ion buffering in solution.

undiluted urine was recorded against deionized water as a reference blank [Figure 2.11 (a)]. The spectrum showed that the sample did not absorb in the region where calcium measurements are carried out. Figure 2.11 (b) shows the spectrum of urine treated with the indicator arsenazo-111. A series of urine samples were analysed for free calcium by SIIM using the indicator arsenazo-111, as follows: to a 2.00mL of urine sample was added 1.00mL of a 2.5 X 10^{-4} M solution of arsenazo-111 and the sample volume made up to 5.00mL with distilled water which had been passed through a mixed bed resin. The absorbance of each solution was then measured against a urine reference solution which was diluted to the same volume. After measuring the first absorbance an increment of 10uL of 0.001M solution of calcium was added and the absorbance remeasured. The concentration of free calcium was evaluated as before.

The results of these experiments are summarized in table 2.11 along with the initial pH of the samples and the mean and standard deviation of each series. The results obtained with the SIIM technique are comparable to the range of pCa reported in the literature (49, 142-143).

The determination of free calcium concentrations by SIIM demonstrates that it can be applied in a variety of sample matrices where the metal ion buffering is unknown and where other methods tend to give unacceptable results.

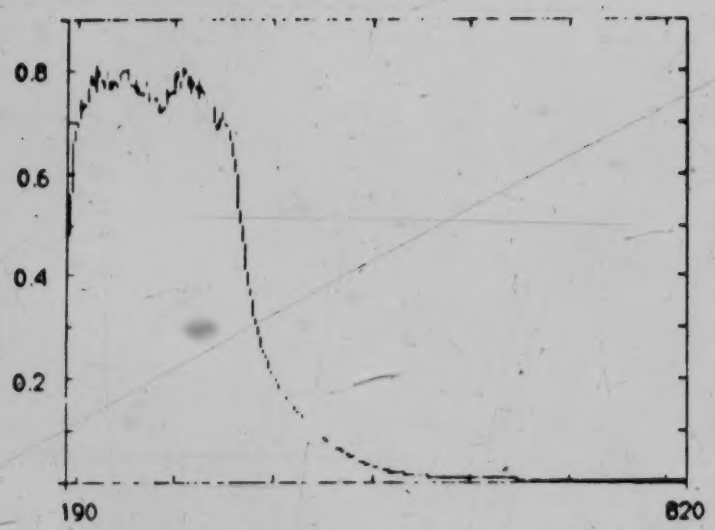


Figure 2.11 (a) Visible absroption spectrum of urine

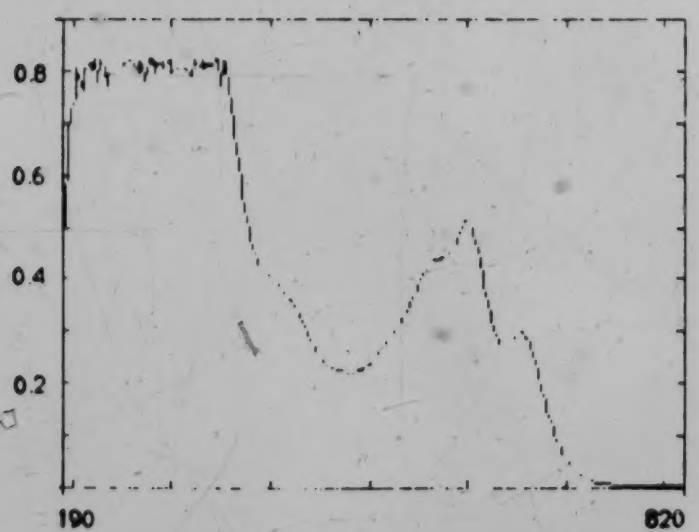


Figure 2.11 (b) Visible absorption spectrum of calcium-arsenazo-111 complex in urine

Table 2.11

Summary of Free Calcium Concentrations Obtained in Urine Samples by SIIM :

Volume of urine sample used	2.00 mL
Arsenazo-III used	1.00 mL of 2.5 X 10^{-4} M
Calcium increment	10 µL 0f 0.001 M

pCa		
Sample # 1	Sample #2	Sample # 3
5.48	5.36	5.17
5.56	5.44	5.11
5.54	5.49	5.01
5.59	5.31	5.07
5.53	5.38	5.12

Average and standard deviations :

Sample 1 : 5.54 (0.0406); Sample 2 : 5.39 (0.070) ; Sample 3 : 5.09 (0.059)

CHAPTER 3

MEASUREMENT OF IONIC CONCENTRATIONS OF IRON (111) BY THE SAMPLE ION INCREMENT METHOD USING XYLENOL ORANGE

3.1 Introduction

The theoretical considerations of metal ion buffering leading to the development of the modified spectrophotometric method called the sample ion increment method (SIIM) were discussed in chapter 2, section 2.1.

In this study iron (111) was selected owing to its importance in various physiological processes (discussed in chapter 1, section 1.1.) and because no method exists to measure the concentration of free (unbound or labile) iron (111) in the presence of bound forms, especially in biological fluids such as blood serum.

In this study xylenol orange was selected as an indicator ligand for the determination of free concentrations of iron (111) by SIIM because it met most closely the requirements imposed on the indicator ligand by SIIM technique as discussed earlier in chapter 2, section 2.2.1. In this study it is shown that purified xylenol orange satisfies the requirements mentioned earlier. Xylenol orange complexes iron (111) selectively at pH values below 2.0, allowing free iron (111) concentrations down to 10^{-6} M to be conveniently measured without interference from other metal ions normally present in biological fluids.

3.2 Determination of Reaction Stoichiometry and Conditional Formation Constant for the Iron (111) - Xylenol Orange Complex

3.2.1. Experimental:

Reagents and Solutions:

Xylenol Orange Solution: The commercially available dye (Fluka Chemicals) was purified by the method of Sato (106), with the only modification that 0.1 M KNO3 was used for elution of the material from the DEAE column (diethylaminoethyl cellulose, DE-23, Whatman Chemicals). Commercial DEAE-cellulose was soaked in de-ionized water for 3 hours, and the slurry was introduced into a 3.5 cm diameter and 20cm long glass tube to give a column of approximately 12cm length. The column was washed with 0.1M HCl, de-ionized water, 0.1M NaOH, de-ionized water, 0.1M NaOH, de-ionized water, 0.1M HCl and de-ionized water, in that order. When a suitable amount of xylenol orange solution is loaded on to the column and eluted with 0.1M KNO_3, three coloured bands appear gradually. These correspond to the compounds cresol red, semi xylenol orange and xylenol orange. The amount of xylenol orange loaded at one time should be less than 0.1mmol per 100 meq of DEAE cellulose for maximum seperation of these compounds. As eluent NaCl has also been proposed by Sata, but KNO_3 was preferred, since all ionic strength adjustments were made using it in subsequent work. The purified xylenol orange was found to have a molar absorptivity of 26,500 under similar conditions (107). Solutions of xylenol orange were prepared fresh daily since they decomposed with time and the decomposition is accompanied by the loss of the metallochromic properties of xylenol orange (108). The stock solutions were 2.75×10^{-4}M in xylenol orange, and the pH was adjusted to 1.25 with HNO_3.

Fe (111) Solution: A stock solution of 0.01 M Fe (111) was prepared from $Fe(NO)_3$ (B.D.H. Chemicals) and the pH adjusted to 1.25 with HNO_3 as before.

KNO_3 Solution: A 1M KNO_3 solution was prepared (B.D.H Chemicals) for ionic strength adjustment.

NTA Solution: A 1.00×10^{-4} M stock solution of NTA (Aldrich Chemical) was prepared to control the degree of iron (111) buffering.

EDTA Solution: A 1.00×10^{-4} M stock solution of EDTA (Fisher Scientific) was prepared for the purpose of controlling the buffering of iron (111).

The distilled water used in preparing all the reagent solutions was passed through a mixed bed ion exchange column and filtered through a 40μm sintered glass filter. All glassware was soaked in $\sim$ 3M HNO_3 overnight and rinsed with deionized water before use.

3.2.2. Optimization of the Experimental Conditions for the Spectrophotometric Measurement of the Fe (111) - Xylenol Orange Complex

Spectra of xylenol orange and Fe(111)-xylenol orange were run against a reference solution containing all the components except the indicator in the case of Fe (111) - xylenol orange and against de-ionized water in the case of indicator alone. The shift in wavelength upon addition of the metal ion, Fe (111), to the indicator solution is big enough so as to be useful for quantitative measurement of Fe (111) without interference from free dye absorbance. The indicator dye shows a maximum absorbance at 440nm while the Fe (111)-xylenol orange complex absorbs at 550nm, as seen

in the Fe-XO spectra in figure 3.1.

The effect of pH on the absorbance of the Fe (111)-xylenol orange complex was studied in an attempt to optimize the conditions for the measurement. A series of solutions were prepared in which the total concentration of iron (111) [C_{Fe}] and the total concentration of indicator xylenol orange [C_{xo}] were kept constant and the pH was varied over a wide range so as to study the effect of pH on the absorbance of the Fe (111)-xylenol orange complex. Each solution contained 12.5 mL of 5.50 X 10^{-5}M solution of Fe (111) and 2.00 mL of 1.0 X 10^{-3}M solution of xylenol orange, with the final concentration of Fe (111) of 2.75 X 10^{-5}M and xylenol orange of 8.0 X 10^{-5}M, at an ionic strength of 0.10 in KNO_3.

3.2.3. Titration Apparatus and Procedure: A microcomputer controlled gravimetric titrator configured in the dual spectrophotometric/ potentiometric mode as described earlier (chapter 2, section 2.3) was used for the photometric titrations. The iron (111) solution (2.0 X 10^{-4}M) was titrated with 2.15 X 10^{-5}M xylenol orange solution. The volumes of the solutions used for titration were 20.00 mL of xylenol orange and 10.00 mL of iron (111) solution. Although only 2.15 mL of iron (111) were required the titration was continued till 8.85 mL of iron (111) solution was added. The measurement of absorbance and pH was carried out at preselected wavelengths of 360, 440, 550 and 700nm after each addition of the xylenol orange titrant to the iron (111) solution. The solution was maintained at a constant temperature of 25C, a pH of 1.25 and an ionic strength of 0.10 in KNO_3. The pH was monitored using a combination pH electrode (Fisher) and an ion/pH meter (Fisher Accumet 521), and the absorbance was monitored by a spectrophotometer (Cary Model 118) using the flow through arrangement discussed earlier in chapter 2, section 2.3. For the mole ratio study a

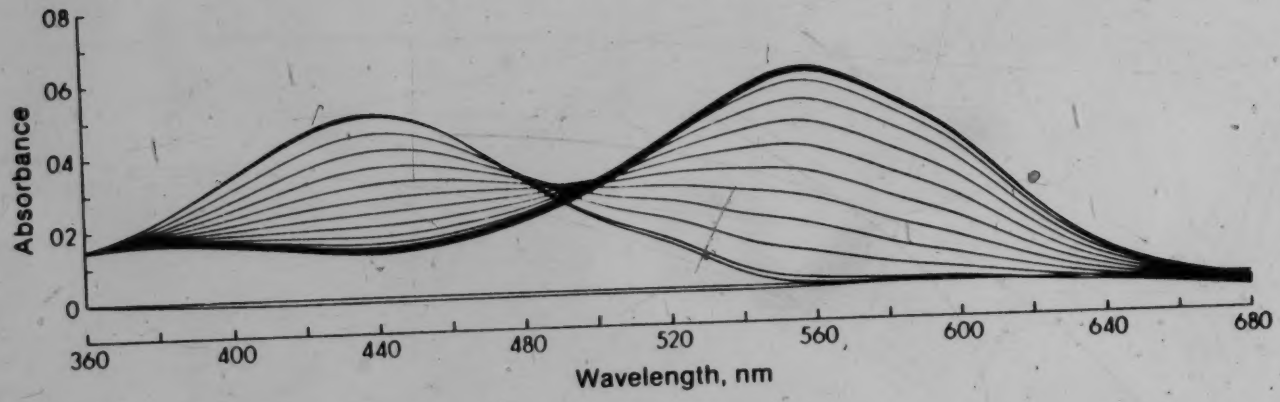


Figure 3.1 Spectra of solution during titration of 100mL of 2.5×10^{-5} M xylenol orange at pH 1.2 and ionic strength 0.1 in KNO_3. Curve with Maximum at 440nm is before the addition of iron(111); curve with Maximum at 550nm is with excess of iron(111)

2.15×10^{-5} M solution of xylenol orange was titrated with a 2.0×10^{-4} M solution of Fe (111) at a pH of 1.25 ± 0.05 and an ionic strength of 0.1 in KNO_3.

3.3 Discussion:

The pH range and the results of this study are summarized in figure 3.2. Figure 3.2 clearly shows that the optimum pH for this system is between 1.0 and 1.30 and that the absorbance decreases sharply outside this range. All the other experiments in this study were done in the pH range of 1.20 to 1.30.

The stoichiometry of the Fe (111) - xylenol orange reaction has been reported by Cheng (107) to be 1:1 only. This was checked by mole-ratio and continuous variation methods. The results of the mole ratio study are shown in figure 3.3 (A) and (B). These results confirm a 1:1 stoichiometry. The method of continuous variation (Job's method) also confirmed the presence of 1:1 stoichiometry for the Fe (111)-xylenol orange complex, figure 3.4. It is evident from the Job's plot that an inflection near molar ratio values of zero and one is absent. This indicated the absence of a 2:2 complex (109). Detection of an inflection near zero or one is difficult if the stability constant is large, but it can be readily observed for intermediate stability constant values. For Fe (111)-xylenol orange, with a stability constant value of intermediate size (see its determination later), the absence of the inflections appears to confirm a 1:1 reaction stoichiometry.

Values of K' for the Fe (111)-xylenol orange system were obtained by both graphical and computational methods. For graphical evaluation the method of Frank and Oswald (110), in which a pair of spectrophotometric titrations are done, was followed. In the first titration the

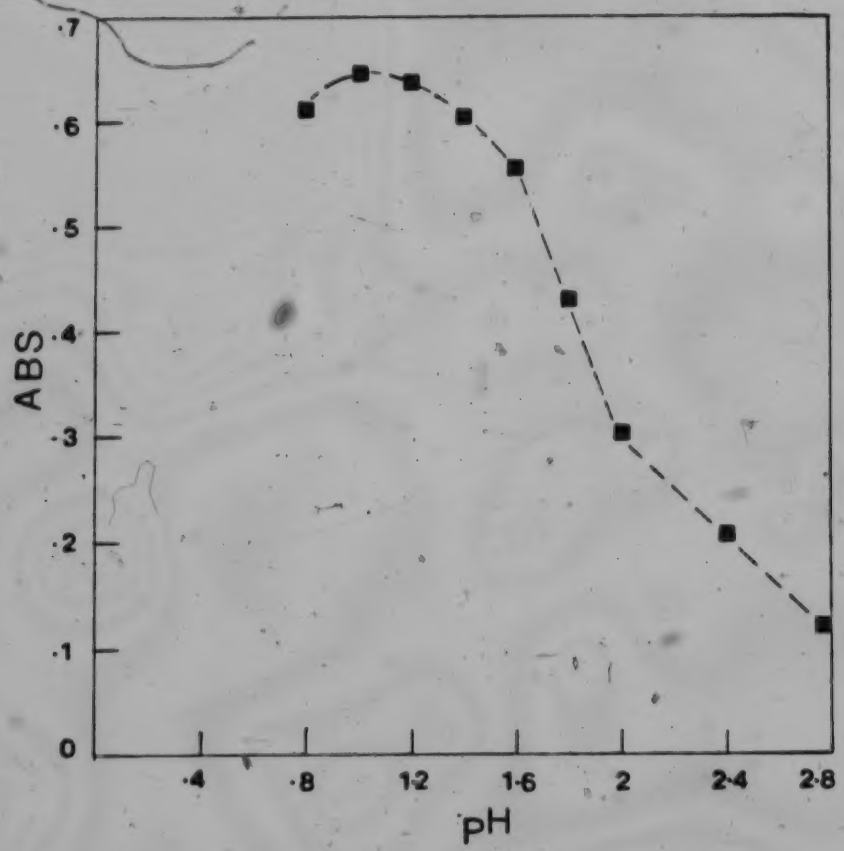


Figure 3.2 Effect of pH on the absorbance of iron(111) xylenol orange complex. Concentration of iron(111) 2.00 X 10^{-4}M and xylenol orange 2.75 X 10^{-5}M

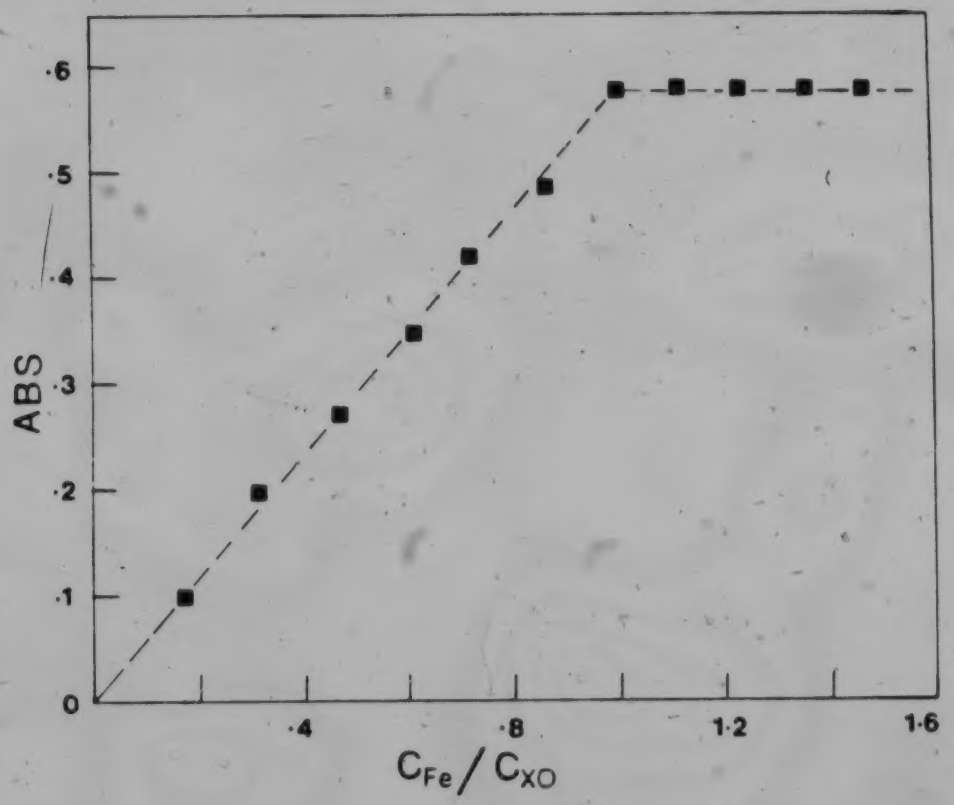


Figure 3.3 (a) Photometric titration of 2.15 X 10^{-5}M xylenol orange with 2.0 X 10^{-4}M iron(111) at pH 1.25 and ionic strength of 0.1 in KNO_3

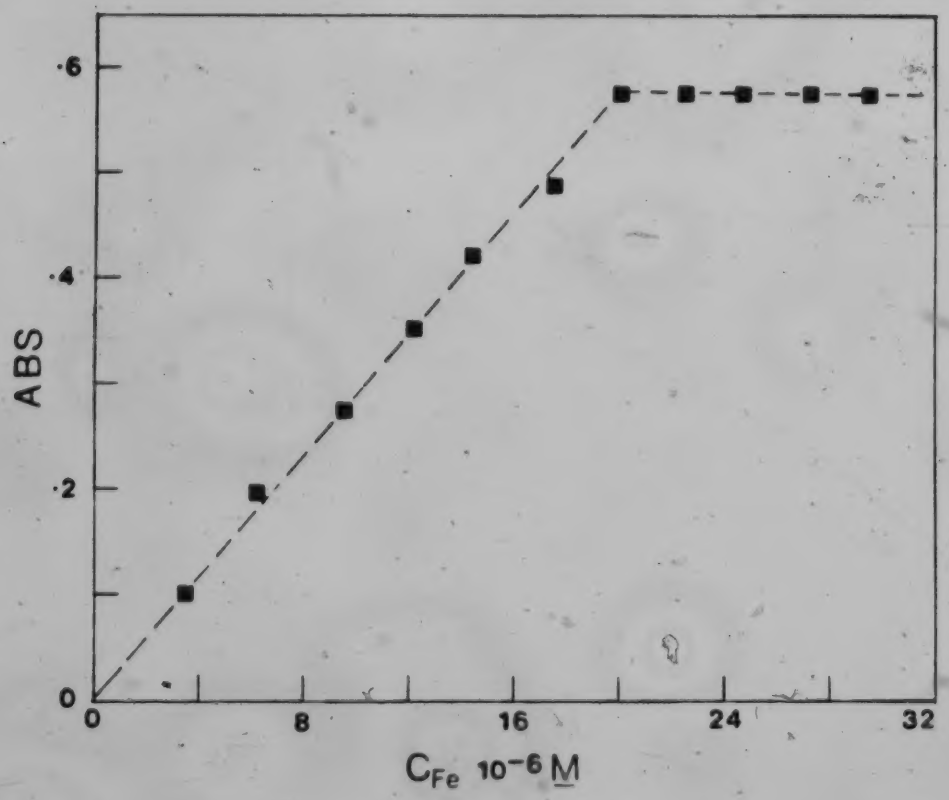


Figure 3.3 (b) Photometric titration of 2.15 X 10^{-5}M xylenol orange with 2.0 X 10^{-4}M iron(111) at pH 1.25 and ionic strength of 0.1 in KNO_3

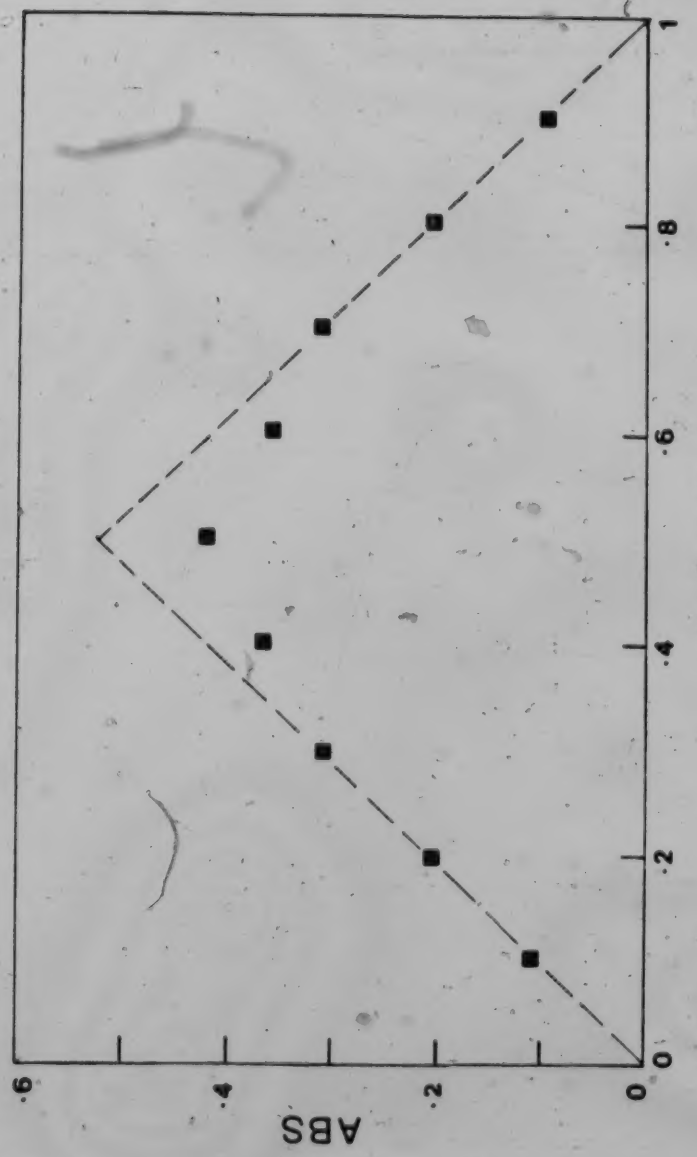


Figure 3.4 Job's Plot

total concentration of the xylenol orange ligand (C_{xo}) is kept constant and the total concentration of Fe (111) (C_{Fe}) is varied; in the second C_{Fe} is kept constant and C_{xo} is varied while all other experimental parameters (pH, ionic strentgh, temperature) remain the same. Data were collected using the gravimetric titrator and the procedure described earlier.

For the reaction (charges omitted for simplicity)

$$Fe + XO \underset{K'}{\rightleftharpoons} FeXO$$

at constant pH and ionic strength, the following relations hold:

$$K' = \frac{[FeXO]}{(C_{Fe} - [FeXO])\ (C_{xo} - [FeXO])} \qquad (1)$$

$$[FeXO]^2 - (C_{Fe} + C_{xo} + 1/K')\ [FeXO] + C_{Fe}\ C_{xo} = 0 \qquad (2)$$

$$[FeXO] = \frac{C_{Fe} \cdot C_{xo}}{C_{Fe} + C_{xo} + 1/K'} + \frac{(C_{Fe} \cdot C_{xo})^2}{(C_{Fe} + C_{x0} + 1/K')^3} + \ldots \qquad (3)$$

Since [FeXO] can be obtained from an absorbance reading at 550nm, then if no other species absorb and Beer's law is followed, for a path of 1cm, A = EC where A = absorbance and E = molar absorptivity at a particular wavelength for a concentration C. Under these conditions:

$$A = E[FeXO] = \frac{E\ C_{Fe}\ C_{xo}}{C_{Fe} + C_{xo} + 1/K'} \qquad (4)$$

Here the second and higher terms in equation 3 have been dropped.

$$\frac{C_{Fe}\,C_{xo}}{A} = \frac{1}{E}(C_{Fe} + C_{xo}) + \frac{1}{EK'} \qquad (5)$$

A plot of $C_{Fe}C_{xo}/A$ against $C_{Fe} + C_{xo}$ yields a straight line of slope 1/E and intercept 1/EK' if only one complex is formed. If more than one complex is formed deviation from a straight line will be observed when either C_{Fe} or C_{xo} is high relative to the other. A plot of experimental data (figure 3.5) shows no significant deviation from linearity, giving a line with an intercept of 8.65×10^{-11} and a slope of 4.248×10^{-5}, from which a log conditional stability constant (K') of 5.69 and a molar absorptivity of 23,600 $mol^{-1}cm^{-1}$ were calculated. The data for the graphical method was also fitted to a nonlinear least squares program based on the Runge-Kutta algorithm (111) written in FORTRAN for an IBM PC microcomputer. This computational approach also gave a value for the log conditional formation constant for Fe (111)-xylenol orange of 5.69 (average of five runs), in agreement with the value obtained graphically. The results obtained for the log conditional formation constant in this study are in acceptable agreement with the value reported by Cheng (107) of log 5.60.

3.4 Determination of Free Iron (111) in Synthetic Samples by the SIIM

As already mentioned in chapter 2, the measurement of free metal concentration by the SIIM involves the measurement of absorbance after the addition of indicator to a solution containing the metal of interest and measuring a second absorbance of the same solution after the addition of an increment of the metal ion of interest. To demonstrate the SIIM technique in measuring free iron (111)

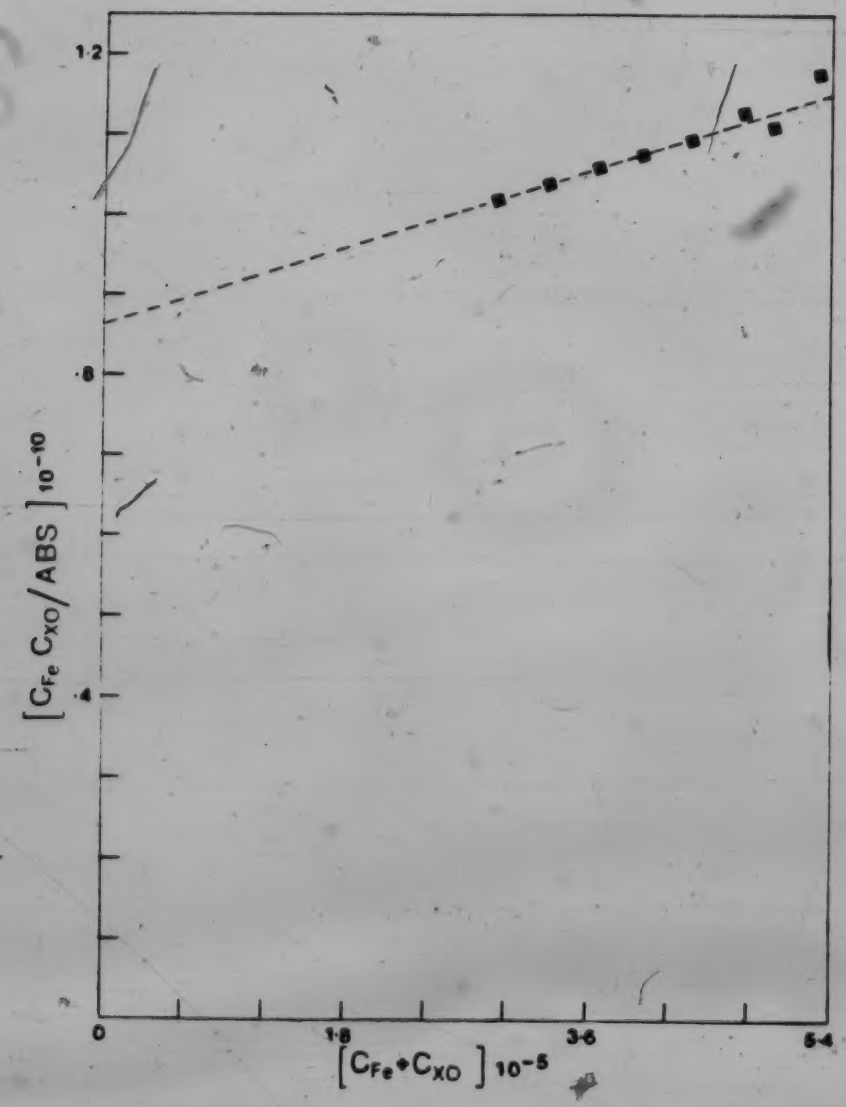


Figure 3.5 Frank and Oswald plot for the determination of conditional formation constants of iron(III)-xylenol orange complex

concentrations two series of synthetic samples were prepared, one in which a buffering ligand was present whose conditional stability constant with iron (111) was high, and a second in which it was intermediate. The ligand chosen for the first series was EDTA, with a log K' value for iron (111) at pH 1.25 and ionic strength 0.1 of 9.2, and for the second NTA, with a log K' value at pH 1.25 and ionic strength 0.1 of 5.9. These values compare with log K' for Fe (111)-xylenol orange at pH 1.25 and ionic strength of 0.10 in KNO_3 of 5.69. It was important to test auxiliary ligands with different K' values to determine whether pFe values experimentally obtained by the SIIM technique agree with the calculated values under differing degrees of metal-ion buffering.

One series of solutions contained varying amounts of EDTA while the concentration of Fe (111) was kept constant (table 3.1); in this way the concentration of free (unbound) iron (111) was varied. To each solution a known amount of xylenol orange was added and the absobrance measured. After measurement a small known increment of iron (111) was added to each solution and the absorbance remeasured.

In a second series of solutions NTA was the auxiliary ligand. Here the total concentration of both NTA and Fe (111) was varied (table 3.2). Each solution was then treated in an identical manner as for the EDTA series.

The date for the SIIM technique were processed using the computer program CFMC. The results obtained for both series of synthetic solutions are listed in tables 3.1 and 3.2. The results show good agreement between the concentration of free iron (111) obtained experimentally and that calculated from the equilibrium data. Therefore the SIIM technique can be applied to the measurement of free iron (111) concentrations over a range of iron (111) buffering.

Table 3.1

Comparision of Calculated and Experimental Values of Free Iron (III) { pFe } Obtained by the Sample Ion Increment Method :

C_{EDTA} , M	pFe		
	Calculated	Experimental	Difference
4.50×10^{-5}	4.76	4.77	+0.01
5.05×10^{-5}	4.92	4.94	+0.02
5.46×10^{-5}	5.10	5.19	+0.09

NOTE : The total concentration of iron (III) { C_{Fe} } , in the above solutions was held constant at 6.25×10^{-5} M throughout .

Table 3.2

Comparision of Calculated and Experimental Values of Free Iron (III) { pFe } Obtained by the Sample Ion Increment Method :

C_{Fe} , M	C_{NTA} , M	pFe		
		Calculated	Experimental	Difference
5.4×10^{-5}	2.01×10^{-5}	4.47	4.56	+0.09
5.0×10^{-5}	2.65×10^{-5}	4.63	4.69	+0.06
4.8×10^{-5}	3.20×10^{-5}	4.80	4.90	+0.10
4.75×10^{-5}	3.16×10^{-5}	4.93	5.09	+0.16
4.20×10^{-5}	3.70×10^{-5}	5.05	5.16	+0.11
4.00×10^{-5}	3.42×10^{-5}	5.24	5.38	+0.14

In these experiments the free iron (111) concentrations ranged from about 13 to 63% of the total.

Table 3.3 summarizes results obtained by the single measurement technique (conventional spectrophotometry) and the SIIM on the same series of solutions. It is clear that the values of pFe obtained with the single measurement technique have more error than the pFe values obtained with SIIM. The results indicate that the SIIM technique achieves the correction needed for the disturbance of equilibria by the single measurement method in the measurement of free iron (111) concentrations in solutions having smaller degrees of metal ion buffering.

3.5 Determination of (Non-Heme) Iron (111) in Blood Serum by SIIM

Having tested the SIIM technique for the measurement of free iron (111) concentrations in synthetic samples, the technique was then applied to blood serum for measuring labile Fe (111) concentrations. A voluminous literature exists on analytical methodology for estimating free iron (111) (non-heme iron) in biological fluids, expecially in blood serum (112-114). Present techniques involve essentially three stages: (1) dissociation of iron from binding proteins such as transferrin by lowering the pH so that the proteins of higher molecular weight precipitate; (2) chemical reduction of the dissociated iron (111) to iron (11); and (3) complexation of iron (11) with a suitable chromogen and absorbance measurement.

Various reagents have been employed for the spectrophotometric measurement of the Fe (11) produced. Also, numerous methods have in common the reduction of Fe (111) to Fe (11) by one of a variety of reducing agents ranging from mild (nitroso-R-salt) to strong (ascorbic acid). The reason for reducing Fe (111) to Fe (11) is to

Table 3.3

Comparision of Free Iron (III) Concentrations Obtained by Single Measurement and the Sample Ion Increment Method :

Free Iron (III) (pFe)		
CALCULATED	Single Measurement	Sample Ion Increment
4.47	4.69	4.56
4.63	4.84	4.69
4.76	4.91	4.77
4.80	5.02	4.90
4.93	5.17	.09
5.05	5.29	5.16
5.10	5.33	5.19
5.24	5.57	5.38

avoid loss of Fe (111) by coprecipitation with serum protein; Fe (11) is not precipitated with the proteins while Fe (111) is (115).

Some methods have attempted to avoid Fe (111) coprecipitation by the use of surface active reagents, or by membrane dialyis as in the autoanalyzer techniques (115-116). The mechanism of the coprecipitation process has not been established, but may be through formation of anions such as $FeCl^{4-}$ that ion pair with large positively charged protein molecules under the high concentrations of HCl employed in the precipitation step. This explanation is supported by Kraus and Moore (117-118).

A disadvantage to the use of a reducing agent is that some iron (111) bound to protein may also be reduced, introducing an error in the value of pFe so determined. Normally the concentration of serum iron is in the range of 80-160 μg/100mL, but the methods employing the reduction of Fe (111) to Fe (11) tend to yield higher results (119).

Some attempts have been made to determine iron (111) in blood serum directly. The methods proposed include a spectrophotometric procedure with thioglycolic acid by Joseph (120) and a kinetic method based on the catalysis by Fe (111) of the oxidation of p-phenetidine with hydrogen peroxide (120-121). Both methods have advantages. The colour developed with thioglycolic acid is not stable with time; the method is not selective, nor is it sensitive enough to measure the low concentrations of iron (111) often encountered. The method based on kinetics is susceptible to a number of interferences. It was therefore considered worthwhile to see whether the SIIM technique could be applied to the direct measurement of free [unbound or non-heme] iron (111) concentrations in blood serum, in an attempt to simplify the methodology of measuring free

iron (111) and perhaps to avoid some of the problems often encountered in the conventional spectrophotometric methods.

3.5.1. Experimental Procedure

Fresh samples of blood serum were taken without addition of anti-coagulant and immediately centrifuged at 3500 rpm for 20 minutes to remove the red blood cells. The supernatant was further centrifuged at 3500 rpm for another 15 minutes to obtain the serum. The proteins were next precipitated by the addition of 500μL of 55% HNO_3 to 5.0mL of blood serum and the clear solution after centrifugation was utilized for Fe (111) determination.

To 1.50 mL of blood serum sample was added 0.50mL of 3.50×10^{-5} M xylenol orange and the absorbance measured in a 1 cm length cell at 550 nm against a blank which had the same volume of blood serum but no indicator ligand. After measurement, 10μL of 7.5×10^{-5} M Fe (111) was added to the sample in the cell, the solution was mixed and the absorbance remeasured. The calculations, performed as discussed earlier, gave a concentration of Fe (111) in the blood plasma of 124 ± 2μg/100mL (average and standard deviation of five replicate determinations). This value is in the range of iron (111) reported in serum, 80-160μg/100 mL. The results may still contain a bias toward lower values owing to possible loss of Fe (111) during the precipitation of proteins. This aspect of the problem was not investigated in this study.

Because of the uncertainty in the other methods, experimental comparisons were not attempted; the results obtained are comparable with values reported in the literature, however.

3.6 Conclusion

The measurement of Fe (111) at pH 1.25 will not measure the free iron (111) concentration present at pH 7.11, the pH of the original sample, because some of the Fe (111) will be displaced from ligands in its labile complexes by protons according to

$$FeL + H^{+} \rightleftharpoons HL + Fe^{3+}$$

Thus the pFe will be lower ([Fe] greater) than if conditions were not perturbed. However, Fe in inert complexes, such as hemoglobin, would not be displaced. This problem is one that all present methods have; all measure iron (111) at pH 2. However, measurement of free (non-heme) iron (111) concentrations by the SIIM solves the problems arising from the use of a reducing agent, and permits a direct measurement of Fe (111) to be made. This increases the precision of the method.

CHAPTER 4

MICROCOMPUTER CONTROLLED DATA-ACQUISITION/PROCESSING OF AN ION-EXCHANGE ATOMIC ABSORPTION SYSTEM FOR DETERMINATION OF FREE METAL ION CONCENTRATIONS

4.1 Theory of Ion-Exchange Equilibrium Method

The ion-exchange equilibrium technique for the determination of free metal ion concentrations involves placing a small quantity of Na^+ - form strongly acidic cation exchange resin in a micro-column and passing the sample solution through it until the total metal concentration in the effluent is identical with that in the column influent. The resin is thus brought to equilibrium with the unperturbed initial sample solution. That is, the sample in contact with the resin at equilibrium has not had any M^{n+} ions removed from labile complexes by the resin, and passage of additional sample solution through the column causes no further variation. The concentration of the free metal ion on the resin is then measured, after elution, by atomic absorption spectroscopy (16,17).

In the application of the ion-exchange equilibrium/atomic absorption (IEE-AAS) technique the following assumptions are made:

Assumption 1. All sample and standard solutions are treated with the same high concentration of an inert electrolyte (e.g. $NaNO_3$) such that $[Na^+]$ is constant for all solutions.

Assumption 2. The total equivalents of resin exchange sites, R^-, are much greater than the equivalents of the sample metal ion, M^{n+}, sorbed onto the resin at equilibrium ($[RNa] \gg [R_nM]$).

Assumption 3. The only species of the sample metal that is absorbed onto the resin is M^{n+}.

The complexation equilibrium for an n-valent ion, M^{n+}, which forms a complex ML_n with ligand L^- is as follows:

$$M^{n+} + nL \rightleftharpoons ML_n \qquad (1)$$

Ligand L^- may be the conjugate base of a weak acid. For simplicity equation 1 is the only complexation equilibrium represented. In principle, any number of different kinds of ligands, and higher metal/ligand ratio species than ML_n, can be accommodated. In the presence of a sodium-form strong acid type cation exchange resin, RNa, the metal ion is also involved in the equilibrium:

$$M^{n+} + nRNa \rightleftharpoons R_nM + nNa^+ \qquad (2)$$

and the corresponding equilibrium constants are

$$\beta = \frac{[ML_n]}{[M^{n+}][L^-]^n} \qquad (3)$$

$$K_{IE} = \frac{[R_nM][Na^+]^n}{[M^{n+}][RNa]^n} \qquad (4)$$

The above equation incorporates concentration terms rather than activities since all measurements are made at constant ionic strength (Assumption 1). The distribution coefficient λ of species M^{n+}, defined as [RnM]/[Mn+], is a constant under the experimental conditions used and is characteristic of the metal.

$$\lambda = \frac{[R_nM]}{[M^{n+}]} = K_{IE} \frac{[RNa]^n}{[Na+]^n} \qquad (5)$$

and on rearrangement,

$$[M^{n+}] = \frac{[R_nM]}{\lambda} \qquad (6)$$

This equation is used to calculate the free concentration of metal ion. The various assumptions and ways to determine the parameters involved in the method are discussed in detail in the discussion section of this chapter.

4.2 Need for Automation of the Ion-Exchange Equilibration Technique

The ion-exchange equilibration atomic absorption (IEE-AAS) technique was developed by Cantwell et al to allow measurement of free, unbound, metal ion concentrations in solutions containing both free and bound forms of a metal. The system described (16) esentially consists of the following steps: Washing, loading and elution of the column, followed subsequently by manual data collection and processing. These operations are labour intensive and time consuming, even though all the steps are performed in a flow through mode (17). A flow chart of the semi-automated system described earlier (17) is shown in figure 4.1. To improve the efficiency of the system for routine analysis the manual operations can be reduced in the following steps: 1. Solution handling needs to be automated. Wash, sample and eluent solutions need to be fed to the column, followed by a second wash. This is labour intensive, but can be done by switching valves so as to regulate the flow of the solutions in the various cycles of the ion-exchange process. 2. Data collection from the atomic absorption spectrometer and subsequent data

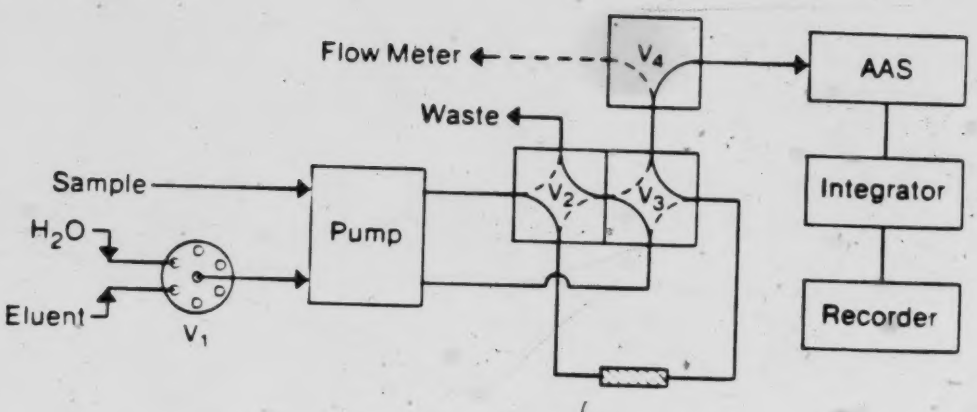


Figure 4.1 Flow chart of the semi automated ion-exchange atomic absorption spectroscopy system for the free metal ion determination; system prior to automation

processing can be performed by a microcomputer.

Due to the above limitations of the manual IEE-AAS system, automation of the system was considered and was carried out with a view to designing a modular integrated system which could overcome the limitations discussed above.

4.2.1 Introduction

At the present time automation of analytical instruments generally consists in coupling the instrument with a mini-or microcomputer to provide control of operation and to perform subsequent data processing and manipulation. Data processing after all the data has been acquired is not as desirable as real time processing, however. Real time processing allows immediate assessment of data quality, including identification of improperly prepared or mixed samples, of instrumental problems, or of procedural errors.

A second desirable feature of an ideal automated system is the possibility of improved sample handling and processing prior to the measurement operations. Many commercial instruments incorporate a turntable-type sample introduction device, but extension of such systems to larger sample volumes and to preliminary sample treatment is difficult. An attractive alternative approach is the use of robotics for sample preparation and handling (122-124). This was the approach selected in this work.

The cost of automating either old or new instrumentation may be prohibitively expensive relative to return if adequate attention is not paid to effective system design. Reports of data-acquisition and processing systems for atomic-absorption spectrophotometers have been made that employ a minicomputer (125) or a microcomputer (126-128). These systems, however, are in general expensive or have

low precision, have not been designed from the point of view of hardware/software cost-benefit analysis, and do not incorporate more than the measurement step under system control.

In this chapter an overall approach based on the system design cycle is presented for the design of a completely automated system for metal ion speciation incorporating ion-exchange to isolate the free metal species and atomic-absorption spectrophotometry for selective measurement.

4.3 Overall System Design

Automation of an analytical instrument by microcomputer control requires a series of decisions regarding computer-instrument interfacing. For the system presented here both the hardware and software components and their interconnections and interactions (ways in which the software controls the hardware) must be specified. Hardware design can be approached by standard design methodology (129); software design is best done in a design language that is as similar as possible to natural language (130). Once the hardware has been purchased or built, and the software written in appropriate language(s), the components can be tested, integrated, and the overall system evaluated (Figure 4.2). Increased software power and flexibility in the larger capacity of microcomputers of today allow consideration of whether certain tasks, such as peak search and peak area integration, are better done by hardware or software. These decisions should be part of the initial integrated design approach.

The first aspect to consider is application of the various parameters of the design cycle of figure 4.2. to the problem of automating the IEE-AAS system. Initially user requirements must be established.

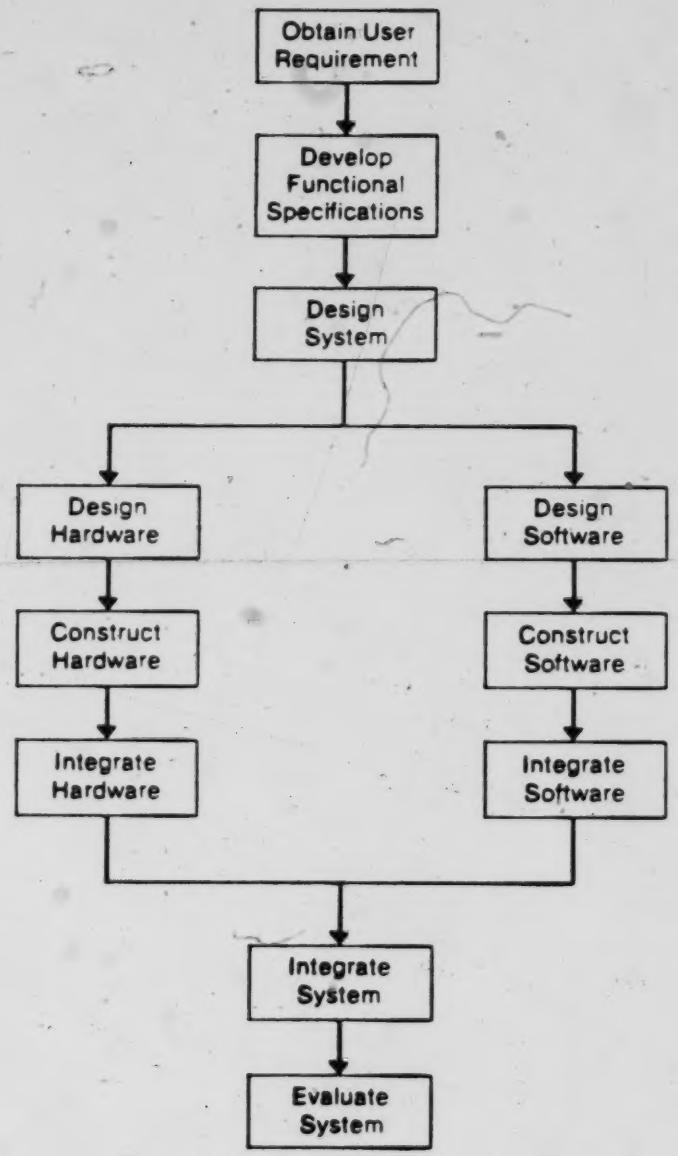


Figure 4.2 Design cycle for a micorcomputer based automated laboratory instrument system

In this case they are:

1. Control the processing and introduction of sample solutions into the system.

2. Operate a valve controller to regulate solution flow through the ion-exchange column and to the atomic absorption spectrophotometer (AAS).

3. Acquire data from the AAS (with real time implementation) and filter the signal if necessary.

4. Process the data acquired from the AAS. This includes the following:
   a. Calculate heights and areas of the eluted peak.
   b. Prepare calibration plots of the standard run(s).
   c. Calculate averages and standard deviations for the replicate runs.
   d. Calculate concentration(s) of unknown samples from the calibrations plots, with attendant statistics.
   e. Perform equilibrium calculations where additional data on ligand concentrations, conditional stability constants of the metal ligand complexes and the acid dissociation constants of the ligands under consideration are available.

5. Provide rapid, simple means of data retrieval for future work.

Next functional specifications for the individual tasks must be provided. These must indicate how to:

   a. Control the robot arm for the sample handling task.
   b. Control the opening and closing of the valves in the various cycles.

c. Acquire data from the AA spectomphotometer.
d. Filter, process and display data acquired from the AA spectrophotometer in real time.
e. Perform statistical and equilibrium calculations on the analytical data.

Lastly, the modules for both the hardware and software components must be established from the functional specifications. Here they comprise the following:

1. Hardware Modules:
   a. Microcomputer.
   b. Robot arm.
   c. Valve controller.
   d. AA spectrophotometer.
   e. Peristaltic pump.
   f. Input interface module.
   g. Input signal conditioning module.
   h. Output devices (printer, plotter, etc.)

2. Software Modules:
   a. Valve controller logic.
   b. Real-time data acquisition.
   c. Real-time praphics display.
   d. Peak height and area measurements.
   e. Statistics calculations.
   f. Equilibrium calculations.
   g. Database management.

4.3.1 Hardware Modules

Once the hardware and software modules have been specified, decisions as to whether to buy or to build the hardware components, and buy or

write the software, must be made. Here careful consideration should be given to the cost and performance of various commercial hardware components before a decision is made. For this application, it was decided to purchase an IBM PC microcomputer with dual double-sided, double density disk drives, 512KB of RAM, and high resolution (720 X 348) graphics capability (Hercules Graphics Card, Hercules Computer Technology, Berkeley, CA), and an interface board with screw terminal panel (DT-2805 interface board and DT-707, screw terminal panel from Data Translation Corp., Marlboro, MA). The IBM PC microcomputer was chosen because it is the most frequently used machine in scientific laboratories and much software is being developed for this machine for laboratory use, e.g. ISSAC (131), ASYST (132), and a control and data - aquisition software library from IBM (133). A detailed review of scientific software available has been recently written by Borman (134) and a quarterly publication from Arizona State University, "Scientific Software Quarterly", provides information and reviews on commercially available scientific software and custom made software developed in various research laboratories. One advantage of chosing the 8088 based IBM PC is that other advanced 8086, 80186 and 80286 based systems are upward compatible with the 8088 based systems; this makes the transition from 8088 based machines much easier.

The DT - 2805 interface board plugs directly into the backplane of the IBM PC microcomputer. It contains 16 single-ended or 8 differential analog input channels which are jumper selectable, along with a wide unipolar or bipolar range (0 to 10 volts or $\pm$ 10 volts), again jumper selectable, and 12 bit ADC resolution adequate for most analytical instrumentation. The board also supports a wide range of programmable amplification (1, 10, 100, 500), has two channels of A/D output with 12 bit resolution and

16 digital 1/0 lines with provision for external clocking and triggering. The optional DT - 707 screw-terminal panel contains a signal conditioning unit. A schematic of the interface board is provided in figure 4.3.

The valve controller unit, built in the electronic shop of the Department of Chemistry, consists of valve driver, control panel, signal conditioning, and power supply sub-units. It was designed to be operable either manually or under computer control. Diagrams of the signal conditioning and valve driver units are given in figure 4.4 (a) and (b). The signal conditioning unit contains a contact bounce eliminator circuit for noise reduction, a two - state latch circuit, a Schmidt trigger circuit, a tristate hex buffer with enable and a four pole double throw switch along with an LED to indicate whether the system is under manual or computer control. The purpose of this unit is to convert the pulse signal from the computer to a level signal for feed to the valve driver sub-unit. The valve driver sub-unit consists of a 74LS123 dual retriggerable one shot chip; circuit I_1 [figure 4.4 (b)] functions on the rising part of the signal slope while I_g functions on the falling part of the signal slope. The timing of these circuits is adjusted to provide 32msec trigger pulses to the 12-Volt dc magnetic latching solenoid valves (General Valve Corp., Hanover, NJ). Although a pulse signal is provided by the computer, the pulse length is too short and erratic to be useful. Hence the signal from the computer was used to initiate generation of the neccessary signal by the valve controller unit.

A variable speed peristaltic pump (Minipuls 2, Gilson, Villiers - Le - Bel, France) and atomic-absorption spectrophotometer (Perkin Elmer, Model 290B) was used. The robot arm (Mitsubishi Electric, Japan, Model RM-101) was interfaced to the parallel part of the multifunction card (AST Research Inc). Solution flow in the system

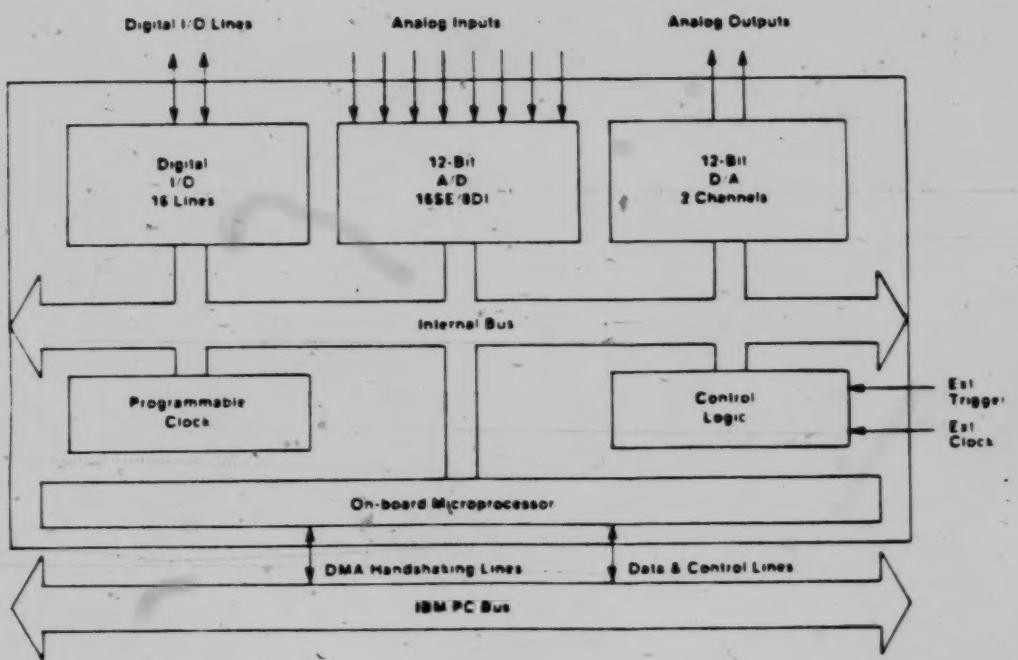


Figure 4.3 Schematic of the interface board DT-2805

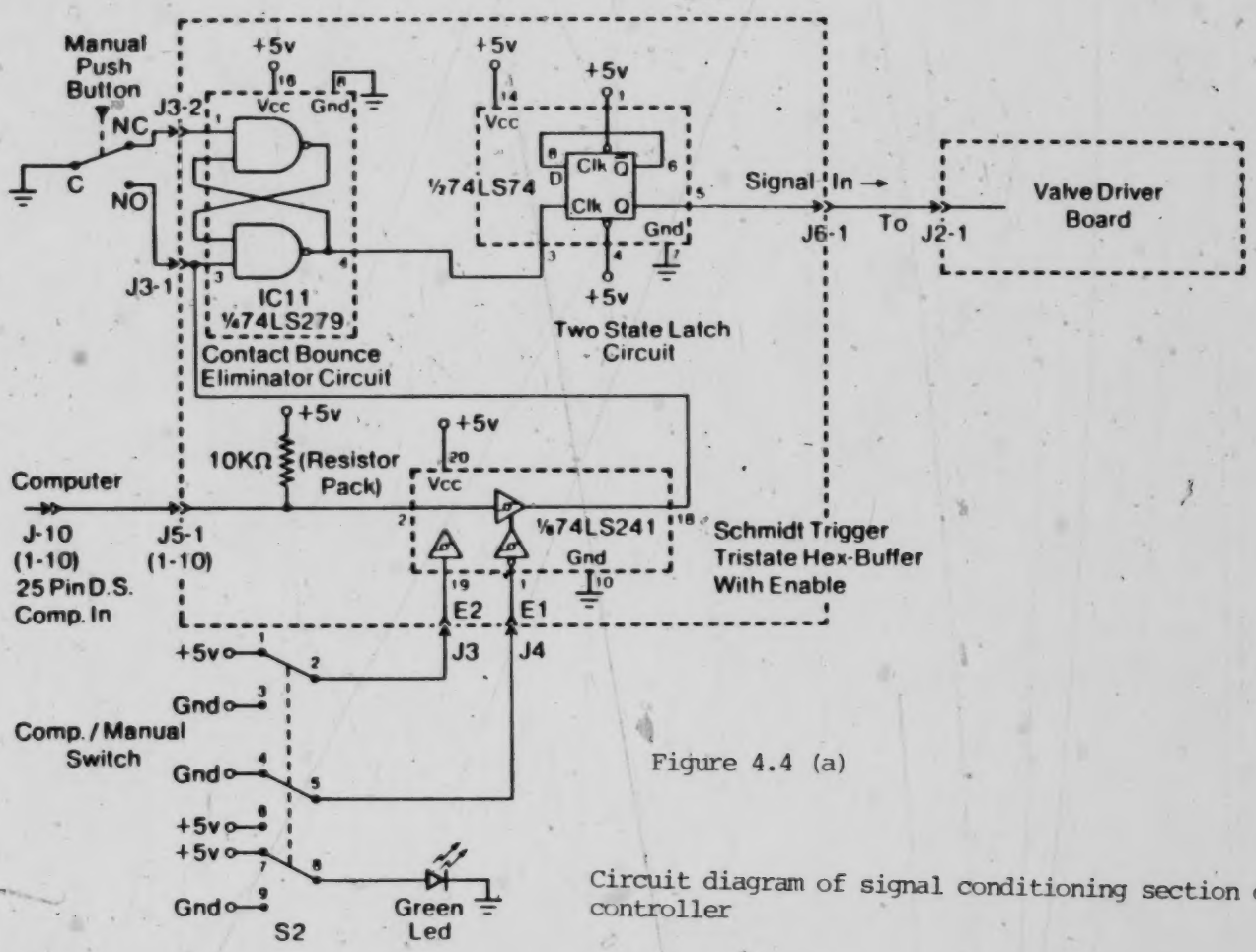


Figure 4.4 (a)

Circuit diagram of signal conditioning section of valve controller

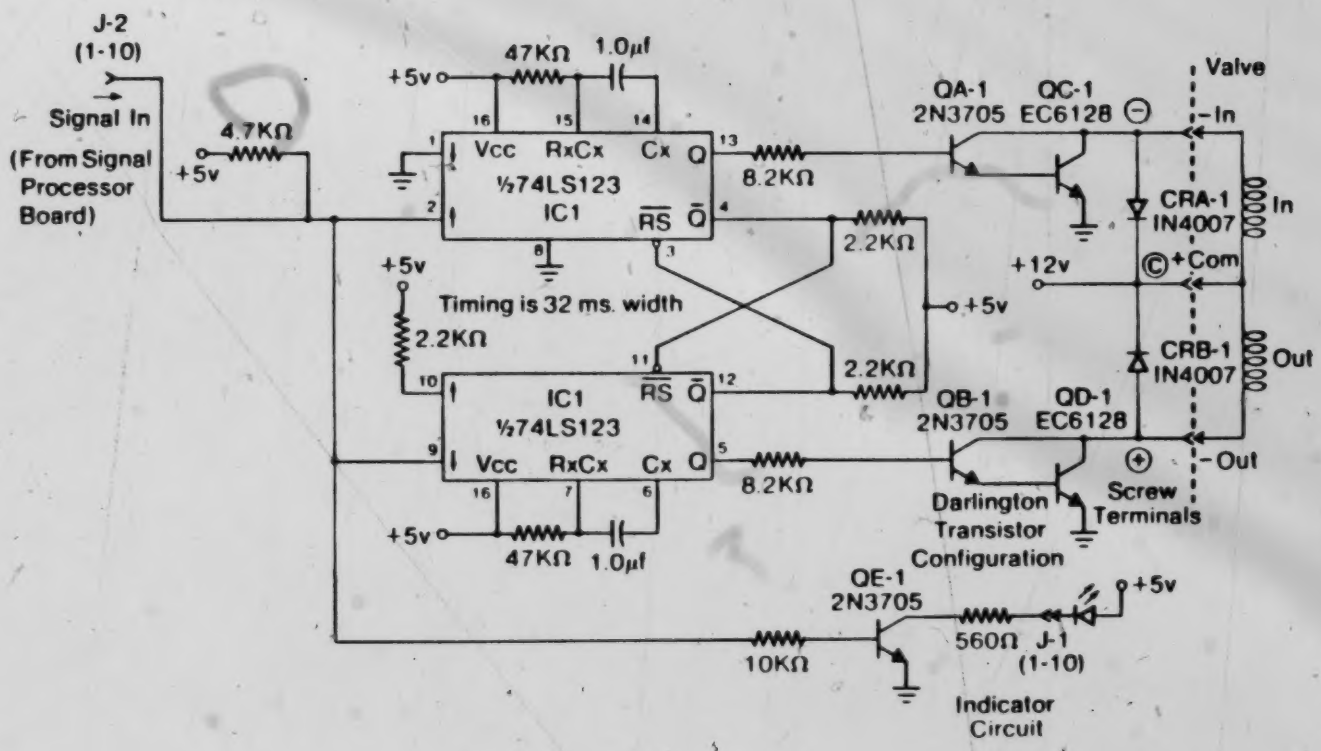


Figure 4.4 (b) Circuit diagram of valve driver section of valve controller

was controlled by a series of solenoid valves V1 to V7 as shown in the integrated hardware system. Valves V1 to V4 are three way valves while a four outlet and one common inlet unit comprises valves V5 to V8 in the system.

Next the hardware modules were integrated into a unit and tested. The interface board DT-2805 and multifunction card were placed in the microcomputer backplane, the valve controller unit connected to the valves and the digital I/O channel of the interface board, and the analog input from the atomic-absorption spectrometer was fed to the ADC channel of the DT-2805. An overview of the integrated hardware system is given in figure 4.5. The solid lines in figure 4.5 indicates the hardware wiring between different components while the dashed lines indicate solution flow.

4.3.2. Software Modules

Once the integration of the hardware modules was complete the software modules, each corresponding to procedures applicable to specific system functions, were developed on the basis of a "top-down" design approach (134). This involves development in the sequence functional specification: system module: procedure. The overall approach is summarized in figure 4.6.

Choice of appropriate programming languages requires careful consideration. The programming languages and operating systems more often encountered in analytical applications have been recently reviewed (135). An important criterion is the rate of data - acquisition and processing relative to convenience of programming; machine language programming is more difficult and time consuming but faster in execution. For this application a hybrid approach was taken i.e., BASIC, which was then compiled using Microsoft

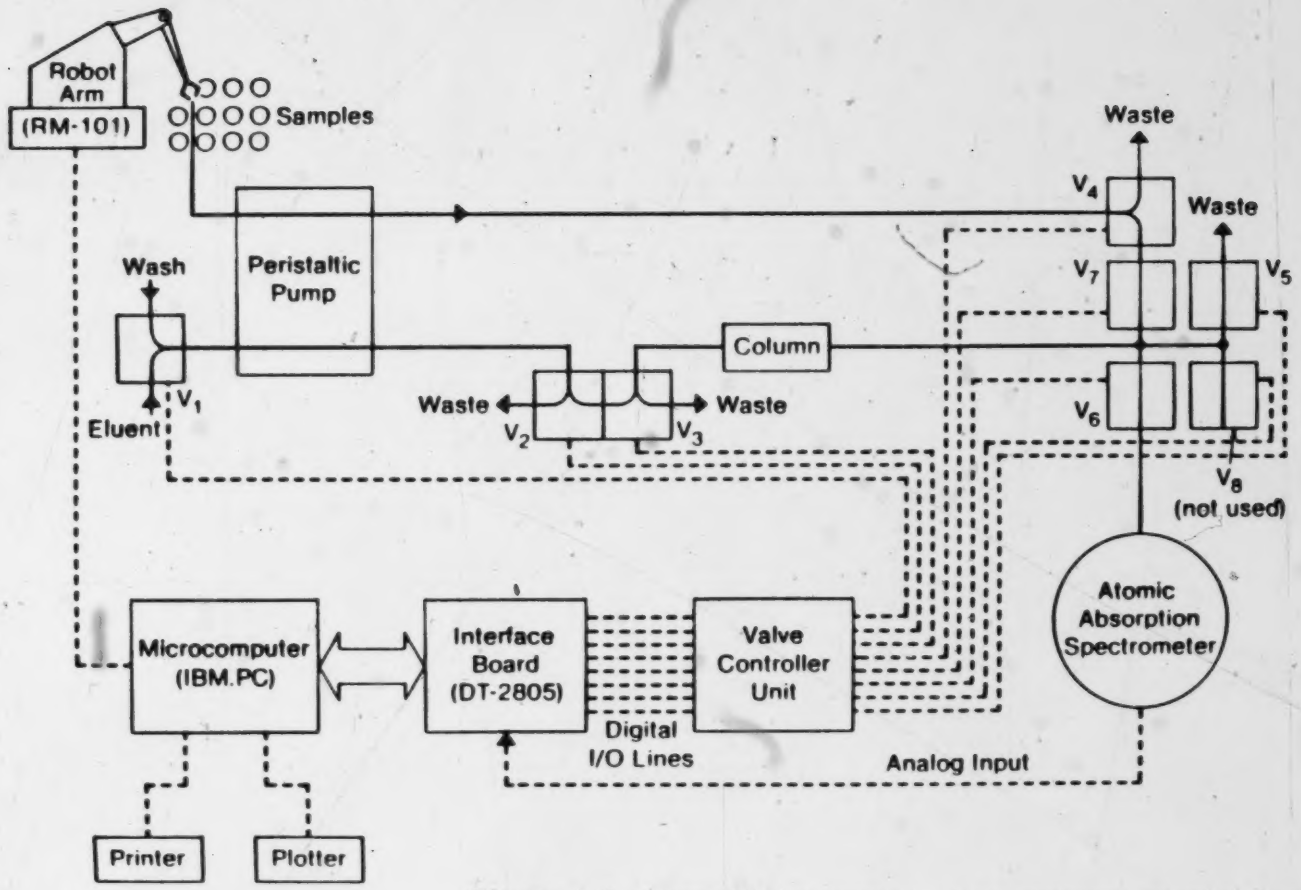


Figure 4.5 Overall integrated (ion-exchange atomic absorption) hardware system

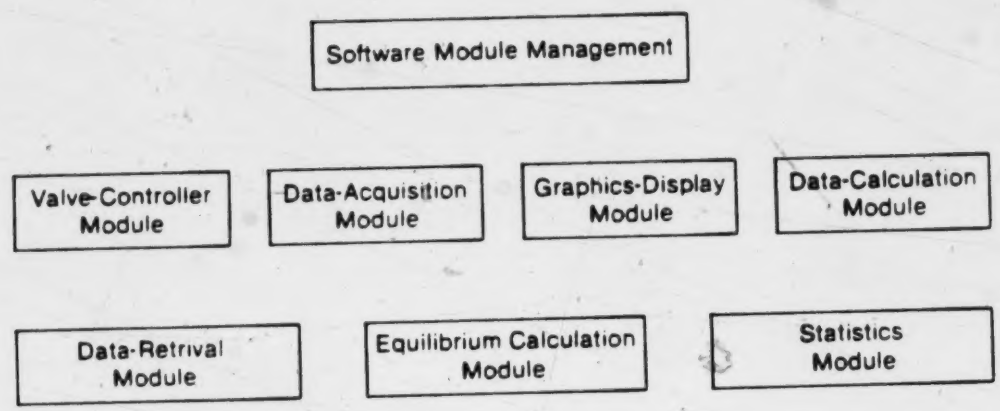


Figure 4.6 Modularization of software units for automated ion-exchange atomic absorption spectroscopy

BASIC complier. 8088 Assembler machine language was used for data-acquisition, valve-control logic and real time graphics display, the remainder was IBM BASIC.

Figure 4.7 shows a flow chart of the module for valve control logic and data-acquisition, while the module for timing and graphics display is shown in figure 4.8. The routine for the calculation of peak area is based on the trapezoidal rule for numerical integration (136). All the software modules were then integrated and tested as before. The valve control logic and corresponding bits required for DI/O lines to control the series of solenoid valves are schematically represented in figure 4.9. The figure also shows the position of the valves in the various cycles of the ion-exchange process.

4.4. Evaluation of the System

Once the integration of the hardware and software modules into a unified package was complete, the system was evaluated in terms of its performance and user friendliness. The concept of system design for hardware and software modules and top down design methodology for software modules makes the entire operation of using this system extremely easy and convenient. When the user inserts the control disk and boots the computer, the first page of the control program showing various options available appears as in figure 4.10. If the experiment is to be performed for the first time then obviously the first option is selected, but if the user intends to review previous experiments then the second option is selected. Option two further prompts the user to input more information about the experiment to be reviewed, as all the experiments are stored and managed under date and experiment number categories in the data-base module. The third option in

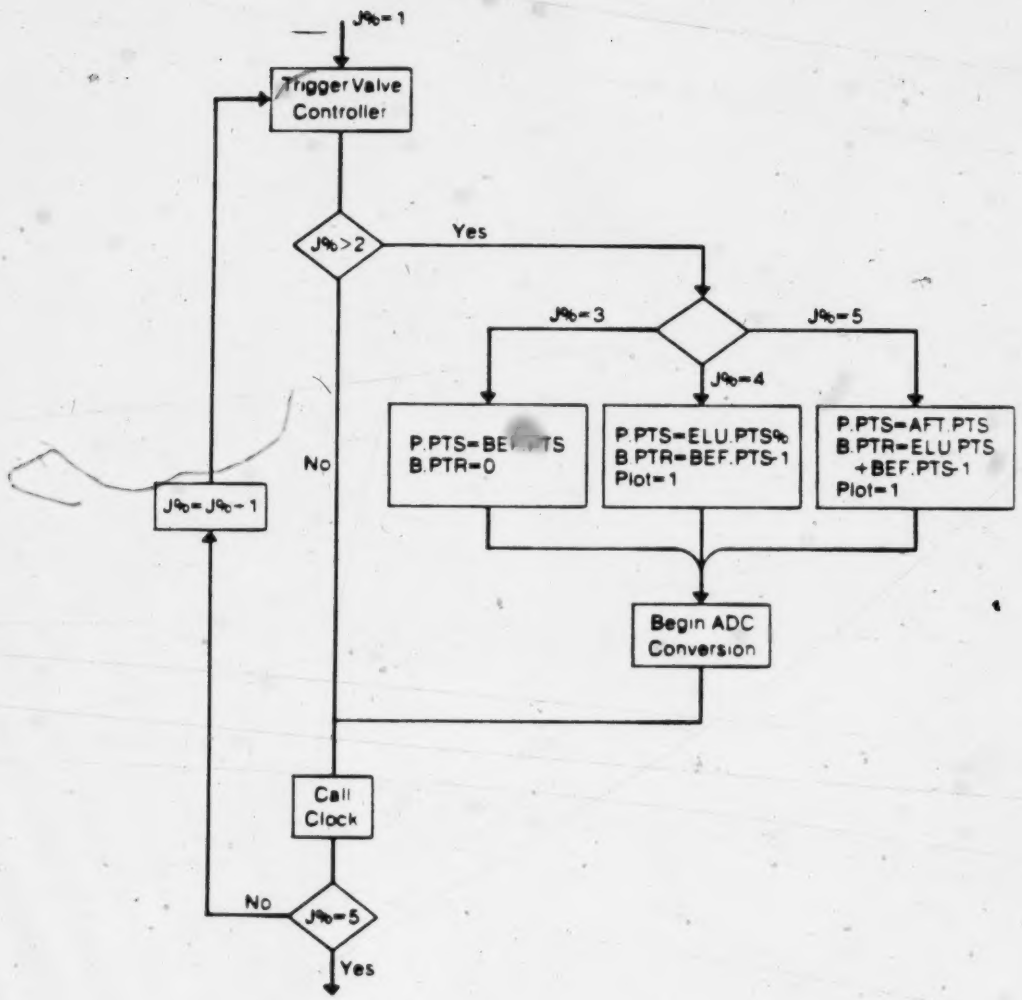


Figure 4.7 Flow chart for control cycle for valve control logic and data-acquisition

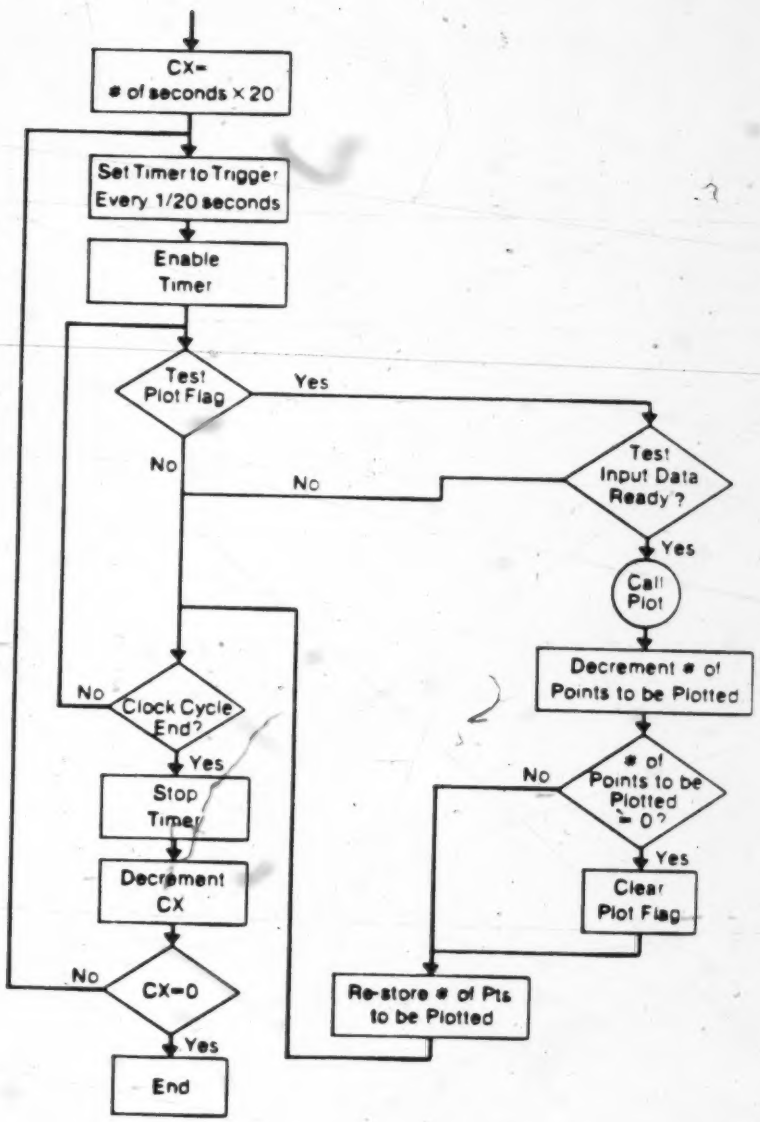


Figure 4.8 Flow chart for control cycle for timing and plotting

	Position of Solenoid Values							Corresponding Bits						
Cycle	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_1	V_2	V_3	V_4	V_5	V_6	V_7
Washing Cycle	on	on	on	off	on	off	on	1	1	1	0	1	0	1
Loading Cycle	on	off	off	on	off	off	on	1	0	0	1	0	0	1
Washing Cycle	on	on	on	off	on	off	on	1	1	1	0	1	0	1
Elution Cycle	off	on	on	off	off	on	off	0	1	1	0	0	1	0
Washing Cycle	on	on	on	off	on	off	on	1	1	1	0	1	0	1

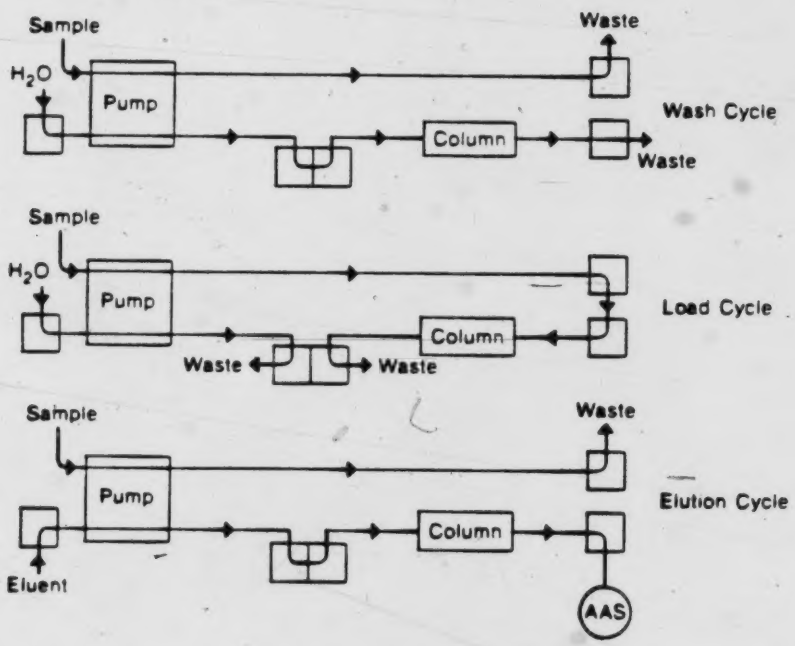


Figure 4.9 Schematic for valve control logic and positioning during different cycles. (See figure 4.5 for identification of valves)

figure 4.10 allows calculation of the concentration of the free metal ion, [M], and free ligand not bound to the metal, [L], provided the total concentration of the metal and the ligand added, along with formation constant data, are available.

If the first option in figure 4.10 is selected, then the computer screen will show the next page as in figure 4.11, which is the experimental set-up record. Here the various parameters are entered by the user. Some of the parameters are meant to be a record and some are essential to control the valves and collect data from the atomic-absorption spectrophotometer. For example, DIO/VALVE CONTROLLER SETTINGS controls the time of the various cycles in the ion-exchange process, and the user has to input time in seconds as shown in figure 4.11. The ANALOG TO DIGITAL CONVERTER SETTINGS control the interface board (DT-2805) and real time data acquisition from the atomic-absorption spectrophotometer. The system as it stands will collect data only on one channel, hence the ADC channel has to be specified 1 at all times. Parameters such as programmable amplification of the input signal and viewing of the signal in either normal or zoomed mode is controlled by the F.S.R. (full scale range) voltage. The COMPUTER CONTROL SETTINGS let the user define the number of times to run the experiments; a maximum of 30 runs per experiment is possible as the system stands now. Because auto storing of data is preferable to the manual option, care has to be taken that a disk is present in the second drive to store the data. The control disk only controls the experiment; all the information which has to be written goes to the second drive containing the data disk.

If an obviously incorrect response is entered by the user the computer beeps and allows the user to enter the correct response. Once all the parameters have been supplied by the user, the computer screen

MICROCOMPUTER CONTROLLED SIGNAL AND DATA PROCESSING FOR

ION-EXCHANGE/ATOMIC ABSORPTION SPECTROMETER

DEVELOPED BY IQBAL TABANI

---------- MAIN MANUAL ----------

1 RUN EXPERIMENT

2 DISPLAY PREVIOUS EXPERIMENTS

3 CALCULATE Kf, [L], [M]

4 STATISTICS

5 CREATE DATA FILES

Enter Procedure #:

Figure 4.10 Main manual; options available

EXPERIMENT SETUP

INSTRUMENTAL SETTINGS:

Element: Ca
Wavelength (nm/A): 422.7
Standard solution concentration (ppm): 0.5 1.0 2.0 3.0 4.0 5.0

DIO/VALVE CONTROLLER SETTINGS:

Washing cycle (sec) = 60
Loading cycle (sec) = 720
Washing cycle (sec) = 60
Elution cycle (sec) = 60
Washing cycle (sec) = 60

ANALOG TO DIGITAL CONVERTER SETTINGS:

ADC channel #(0-7) = 1
Sampling frequency (pts/sec) = 20
Programmable gain (1, 10, 100, 500) = 100
Maximum F.S.R. voltage (5/10)V = 10

COMPUTER CONTROL SETTINGS:

Experiment results storing (Auto,Manual): A
Number of runs: 2

ANY CHANGE NEEDED?

Figure 4.11 Experimental setup record for IEE-AAS

displays the next page. This page controls the initialization of the various solenoid valves, as shown in figure 4.12.

After all the information is entered and the experiment is run, the data acquired can be reviewed on the screen. An example of the data acquired for one sample is provided in figure 4.13 (a) and (b). Once data acquisition is over the computer returns to the first page (figure 4.10) once again. The user can review the experiment input parameters, or perform another run by repeating the above mentioned steps.

Results of studies carried out using this system are discussed in chapter 5.

LIGHT INDIC.	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
ON/OFF (1/0)	1	1	1	1	1	1	1	1	1	1

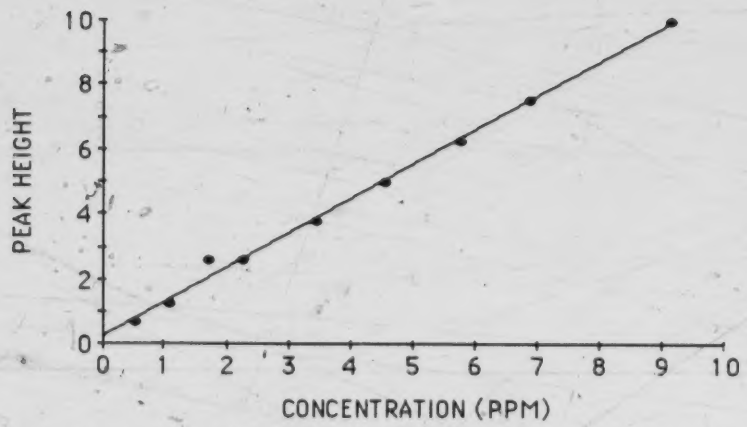
ENTER VALVE CONTROLLER LIGHT INDICATOR'S VALUES

ON = 1
OFF = 0

VALVE CONTROL INDICATORS SHOULD ALL BE OFF NOW

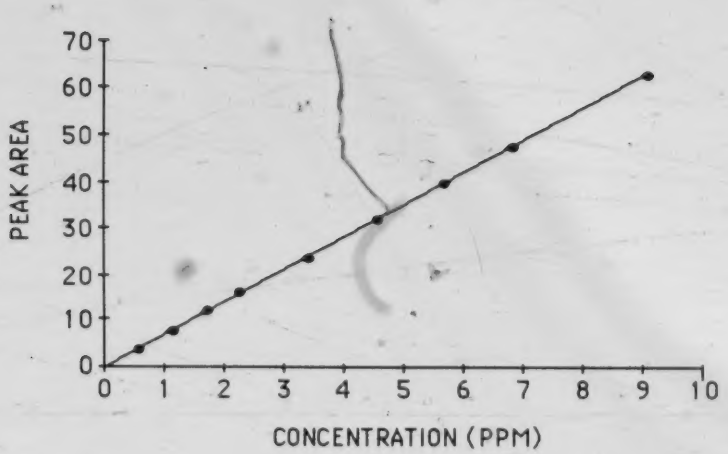
ENTER Y(ES), N(O) OR Q(UIT)

Figure 4.12 Setup for initialization of solenoid valves



REGRESSION EQUATION: Y=0.1477013 + (1.197119)X
COEFF. DET = 0.9958495 COEFF. COR = 0.9979226
STD. DEV. EST = 0.2080343

Figure 4.13 (a) Calibration plot for free calcium; peak height against concentration



REGRESSION EQUATION: Y=-8.460151E-04 + (7.749278)X
COEFF. DET. = 0.9999981 COEFF. COR. = 0.9999991
STD. DEV. EST = 2.770016E-02

Figure 4.13 (b) Calibration plot for free calcium; peak area against concentration

CHAPTER 5

MEASUREMENT OF FREE CALCIUM CONCENTRATIONS BY ION-EXCHANGE EQUILIBRIUM/ATOMIC ABSORPTION SPECTROSCOPY (IEE-AAS)

The results of measuring the free concentration of calcium in various synthetic samples and in urine samples, using the automated ion-exchange equilibration/atomic-absorption spectroscopy system described in the previous chapter, are presented in this chapter. Experiments were undertaken to characterize the IEE-AAS technique for the determination of free calcium concentration in terms of effect of (a) flow rate of solution on the A.A. spectrophotometer signal, (b) time required to attain equilibrium (equilibration time or breakthrough time) for columns containing various amounts of resin and (c) the effect of variation in pH.

5.1 Experimental

Reagents and Chemicals:

Citric Acid: A stock solution of 0.01 M was prepared. (Caledon Labs, Ontario.)

Tartaric Acid: A stock solution of 0.01 M was prepared. (Terochem, Edmonton.)

Sodium Hydrogen Phosphate: A stock solution of 0.01 M was prepared. (B.D.H. Chemicals, Montreal.)

Oxalic Acid: A stock solution of 0.01 M in 1L was prepared. (Fisher Sci. N.J.)

NTA: A stock solution of 0.001 M was prepared. (Aldrich Chemical Company, Milwaukee. Wis.)

Other reagents and procedures for solution preparation were as discussed in chapters 2 and 3. The automated system for measurement was that described in chapter 4.

5.2 Characterization of the IEE-AAS Technique:

To study the effect of flow rate on the AA signal, a known concentration of calcium solution (2 ppm) was pumped directly into the A.A. spectrophotometer by connecting the peristaltic pump tubing to the nebulizer tubing. The pumping rate was then varied over the range of 1 to 9 mL per minute. The results are shown in figure 5.1 which is a plot of relative sensitivity of the AA signal at various flow rates of solution into the nebulizer. It is clear from the plot that the AA signal becomes constant above a pumping rate of 6.00 mL per minute. Hence a flow rate of 7.25 $\pm$ 0.06 mL, on the maximum sensitivity plateau, was therefore employed in subsequent work.

To determine the effect of resin quantity on equilibration time, columns of different sizes containing 5, 10, 50 and 100 mg of strong cation exchange resin (Dowex 50W-X8) were prepared by grinding to pass a 300-mesh sieve, packing into Teflon tubing and inserting a sintered glass frit at both ends. A calcium solution of known concentration, in 0.10 M KNO_3 and pH adjusted to 7.04 $\pm$ 0.05 with MOPS buffer, was pumped through the column (loading step) for various time periods. After each loading the column was washed and eluted into the AA instrument. The loadings were done in order of increasing time till the AA signal became constant. A constant signal showed that the concentration of the influent

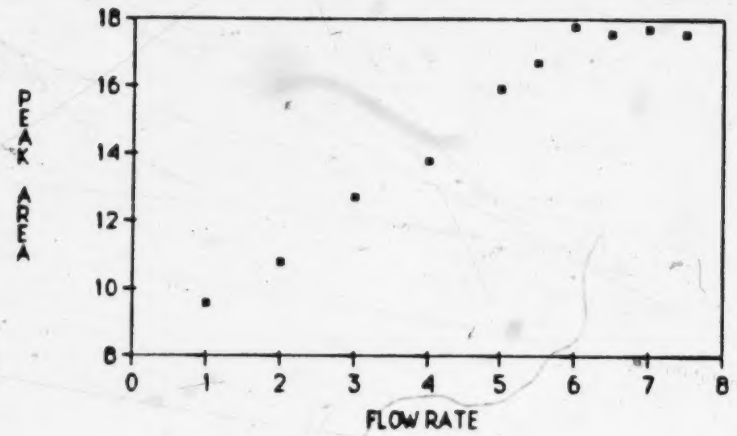


Figure 5.1 Relative sensitivity of the AA signal at various flow rates of solution into the neublizer [C_{Ca} = 2mg/L]

calcium was identical to the effluent and indicated the attainment of equilibrium. Different sizes of columns were used to investigate the time required in each case so that columns could be selected with shorter equilibration times to speed up the method for routine analysis. Figure 5.2 shows the equilibration times required for columns of different sizes. On the basis of this study a column size containing 5.0 mg of resin was selected for subsequent work employing A.A. spectroscopy as the detection technique.

The effect of varying the pH of calcium solutions of known concentration in 0.10 $\underline{M}$ KNO_3 is shown in figure 5.3. It is clearly evident from the figure that variation of the pH over the range of 6 to 9 has no significant effect on the free calcium concentration, so long as no ligand is present in the solution. That is, the equilibrium between Ca^{2+} in solution and on the resin is not affected over the pH range studied.

The influence of ionic strength and K^+ concentration on the measurement of free calcium concentration was investigated as follows. A series of calcium solutions of known concentration (1 ppm) in 0.10, 0.20, 0.25, 0.30, 0.35 and 0.40 $\underline{M}$ KNO_3 were prepared. The pH was kept at 7.03 $\pm$ 0.05 with MOPS buffer in each case. These solutions were then loaded on a 5.00 mg resin column for 11 minutes and then eluted with 3 $\underline{M}$ HNO_3. The equilibration time and calcium concentrations for these solutions varied considerably owing to the increased competition by K^+ for resin sites on increasing the total $[K^+]$, as shown in table 5.1. The effect of ionic strength on the measurement of free calcium ion concentrations is also important (see section 5.5). Thus the combined effect of ionic strength and increased concentration of K^+ in the solutions influences the uptake of Ca^{2+} by the resin and hence the signal of elution varies for the solution of same calcium concentration as

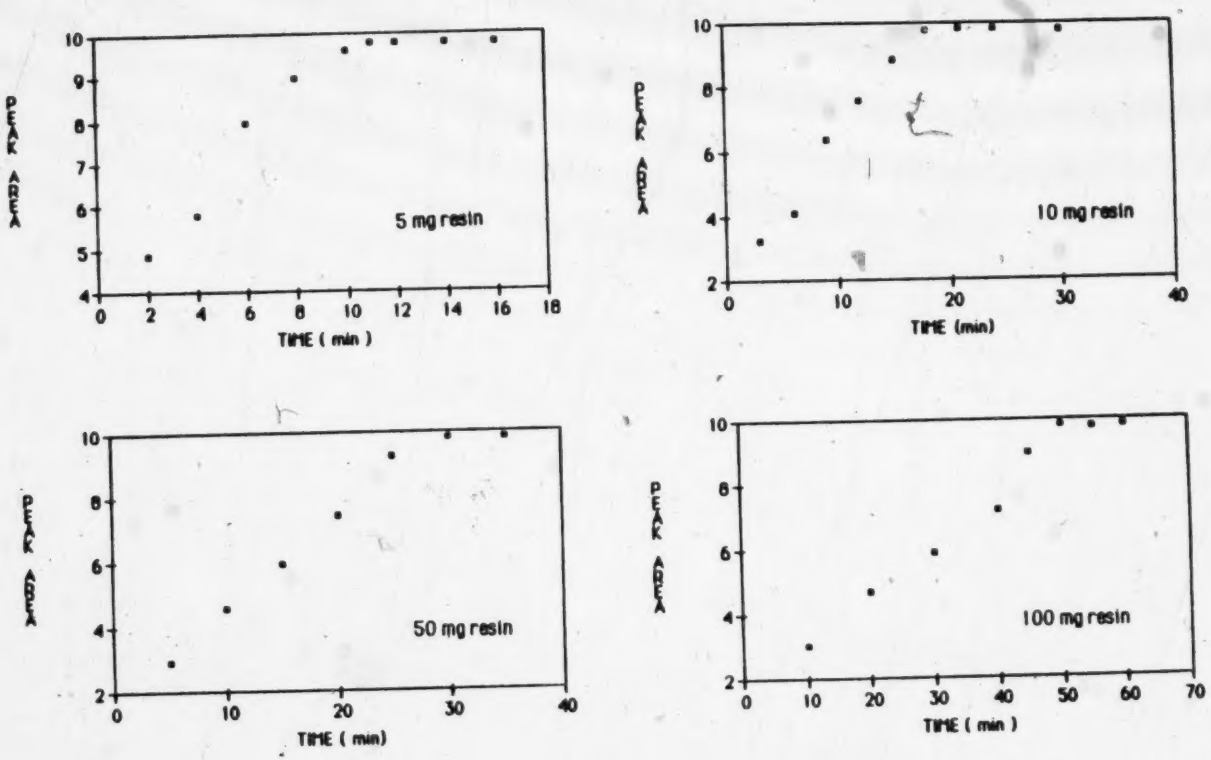


Figure 5.2 Equilibration time required for columns of different sizes $[C_{Ca} = 1 mg/L]$

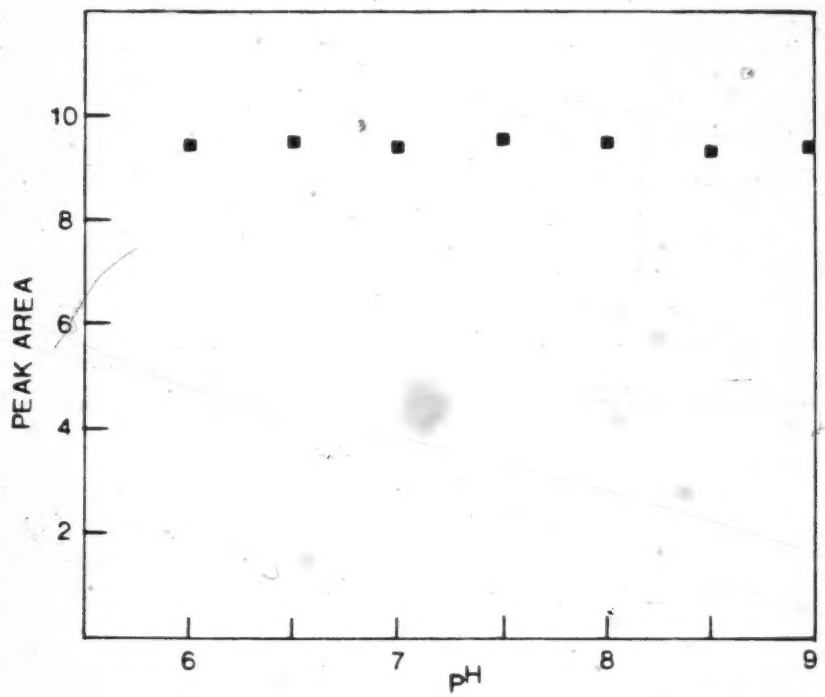


Figure 5.3 Effect of varying pH on the uptake of calcium by resin [C_{Ca} = 1mg/L]

shown in figure 5.4. Each point in the figure was obtained in an experiment where the resin was brought to equilibrium with the calcium solution according to table 5.1.

5.3. Determination of Free Calcium Concentrations in Synthetic Samples by IEE-AAS

Various synthetic samples were prepared in which the total concentration of the calcium was kept constant and the concentration of the ligand added varied so as to vary the degree of calcium ion buffering and thereby the concentration of free calcium. The ligands selected were NTA, citrate, tartrate, phosphate and oxalate for the purpose of preparing the synthetic samples. These were chosen (except NTA) because of their presence in urine. The samples were held at pH 7.02 $\pm$ 0.05 with MOPS buffer and at μ 0.10 with KNO_3.

Effect of Citrate

A series of solutions were prepared in which the total concentration of calcium (C_{Ca}) was kept constant at 1.0×10^{-5} M and the total concentration of the added ligand, citrate, was varied as shown in table 5.2. The pH and the ionic strength of these solutions were kept constant at 7.04 $\pm$ 0.05 (MOPS) and 0.10 (KNO_3). These solutions were loaded on a 5 mg resin column for 11 minutes and eluted with 3 M HNO_3. The results obtained are plotted in figure 5.5. It is evident from the figure that good agreement exists between the values of free calcium obtained using the formation constant data (140) and those obtained experimentally.

The effect of pH on the calcium-citrate system was also investigated. A series of solutions were prepared in which C_{Ca} was kept constant at 1.0×10^{-5} M and the total concentration of the added ligand,

Table 5.1

Effect of Ionic Strength on Equilibration Time :

Column size : 5 mg resin column

C_{Ca} : 1 ppm

Ionic Strength (NaCl)	Equilibration Time (minutes)
0.10	11
0.2 0	9
0.25	8.6
0.30	7.7
0.35	6.9
0.40	5

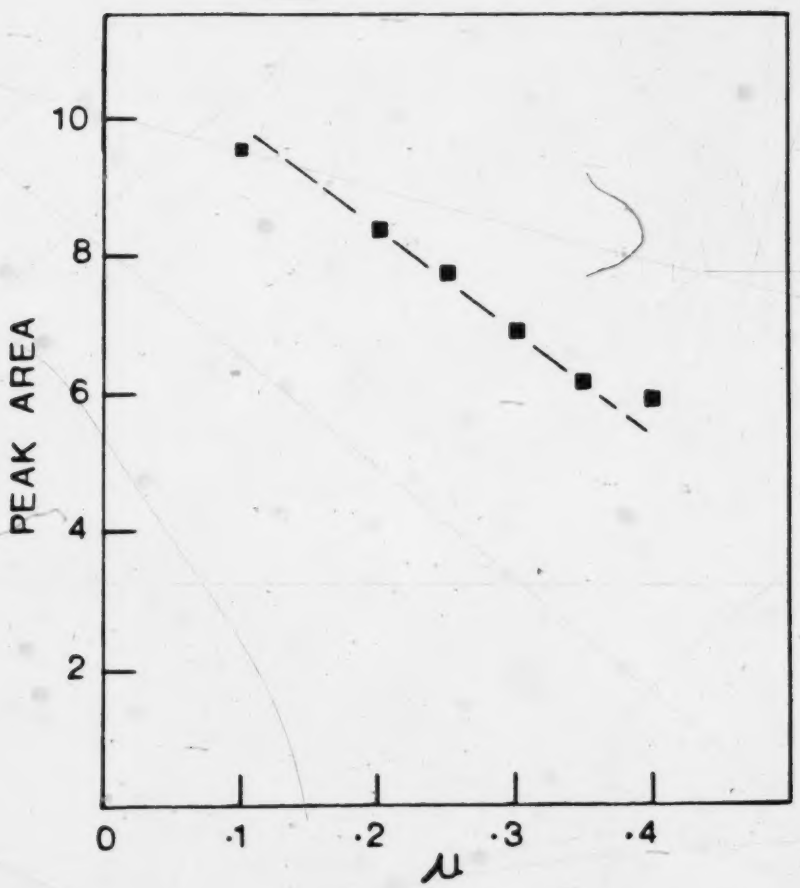


Figure 5.4 Combined effect of increased $[K^+]$ and ionic strength on the uptake of calcium by resin

Table 5.2.

Scheme for the Preparation of Synthetic Samples :

Concentrations Used *					
C_{Ca}	$C_{Citrate}$	$C_{Tartrate}$	$C_{Phosphate}$	$C_{Oxalate}$	C_{NTA}
1.0×10^{-5}	2.5×10^{-4}	2.5×10^{-3}	4.0×10^{-4}	2.0×10^{-4}	2.0×10^{-7}
	5.0×10^{-4}	5.0×10^{-3}	6.0×10^{-4}	4.0×10^{-4}	4.0×10^{-7}
	7.5×10^{-4}	7.5×10^{-3}	8.0×10^{-4}	6.0×10^{-4}	6.0×10^{-7}
	1.0×10^{-3}	1.0×10^{-2}	1.0×10^{-3}	8.0×10^{-4}	8.0×10^{-7}
		1.25×10^{-2}	1.2×10^{-3}	1.0×10^{-3}	1.0×10^{-6}
		1.5×10^{-2}	1.4×10^{-3}	1.2×10^{-3}	1.2×10^{-6}
		1.75×10^{-2}	1.6×10^{-3}	1.4×10^{-3}	
		2.0×10^{-2}		1.6×10^{-3}	

* All concentrations in M .

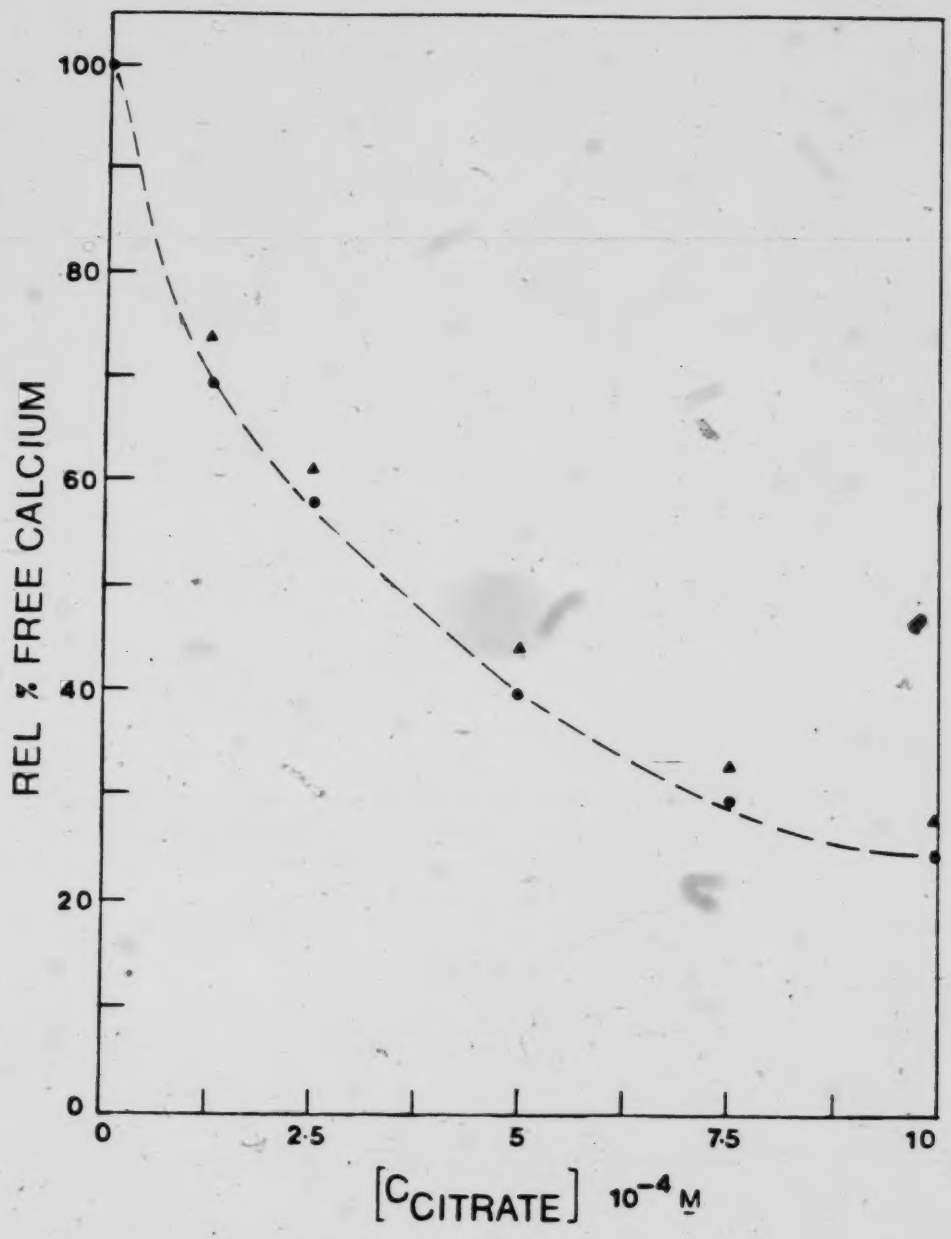


Figure 5.5 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAs, in presence of citrate

$C_{Citrate}$, was also kept constant at 5.0×10^{-4} M. The pH values of these solutions were varied over a wide range from pH 6.00 ± 0.05 to 9.00 ± 0.05 in increments of 0.5 ± 0.05 pH units, keeping the ionic strength constant. These solutions were analyzed as before and the results obtained are plotted in figure 5.6. From the figure it is clear that the concentration of free calcium varies considerably when the pH is low, but above pH 6.5 it is not sensitive to pH cariation. Calculated values of free calcium concentrations from the formation constant data (140) are in excellent agreement with those obtained experimentally.

Effect of Tartrate

The synthetic samples contained a constant concentration of total calcium (1.00×10^{-5} M) and the concentration of the added ligand, tartrate, was varied as shown in table 5.2. The pH and the ionic strength of the samples were kept constant as before. The sample solutions were loaded onto a 5 mg resin column for 11 minutes and eluted with 3 M HNO_3. The results obtained are plotted in figure 5.7. Excellent agreement exists between the values of free calcium concentration obtained experimentally and those calculated from stability constant data (140).

A pH variation study also carried out on the calcium-tartrate system. A series of solutions were prepared in which C_{Ca} and $C_{Tartrate}$ were kept constant at 1.00×10^{-5} M and 1.50×10^{-2} M, while the pH of the samples was varied from 6.00 ± 0.05 to 9.00 ± 0.05 in increments of 0.5 ± 0.05 pH units. The ionic strength was kept constant. The sample solutions were analyzed as before; the results obtained are plotted in figure 5.8, along with the values of free calcium concentration calculated from formation constant data (140).

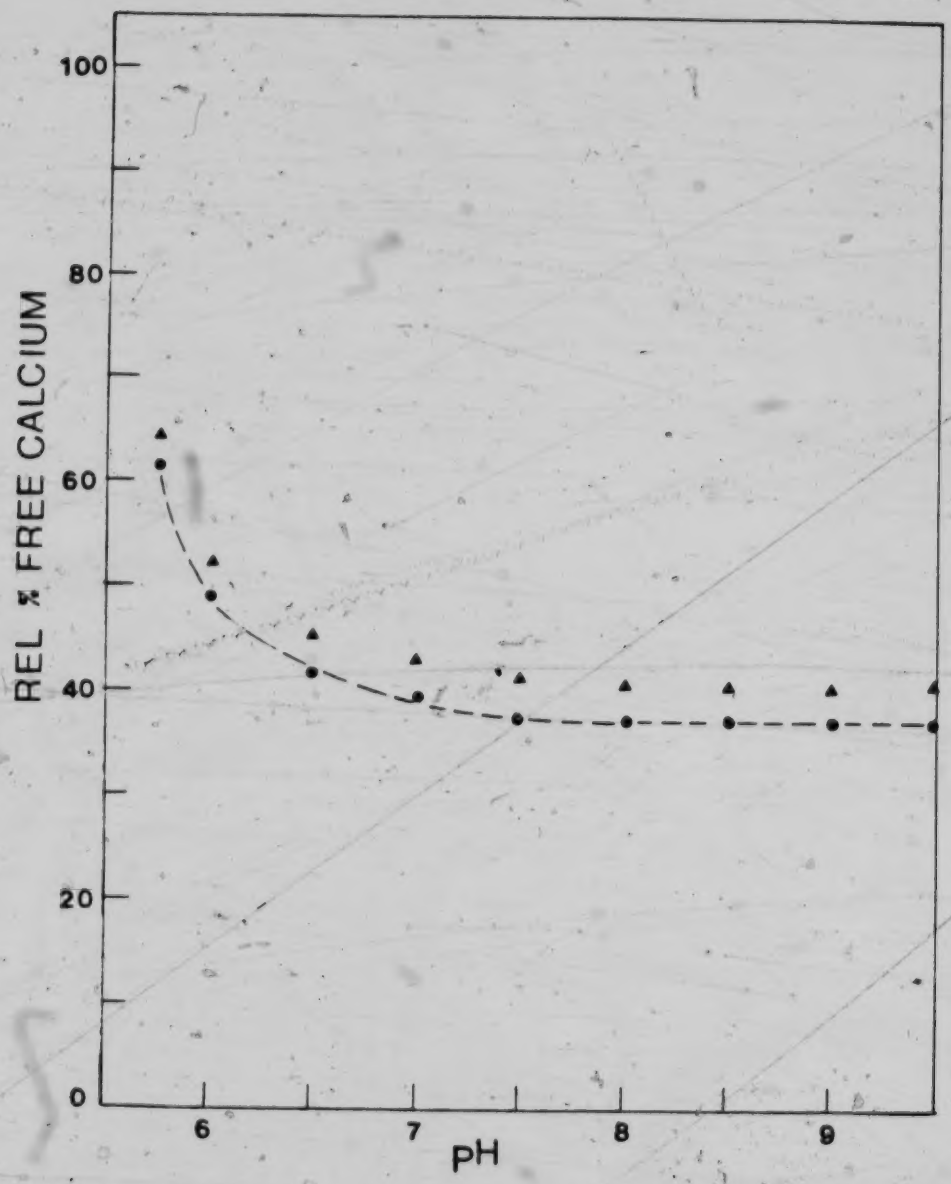


Figure 5.6 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-citrate system

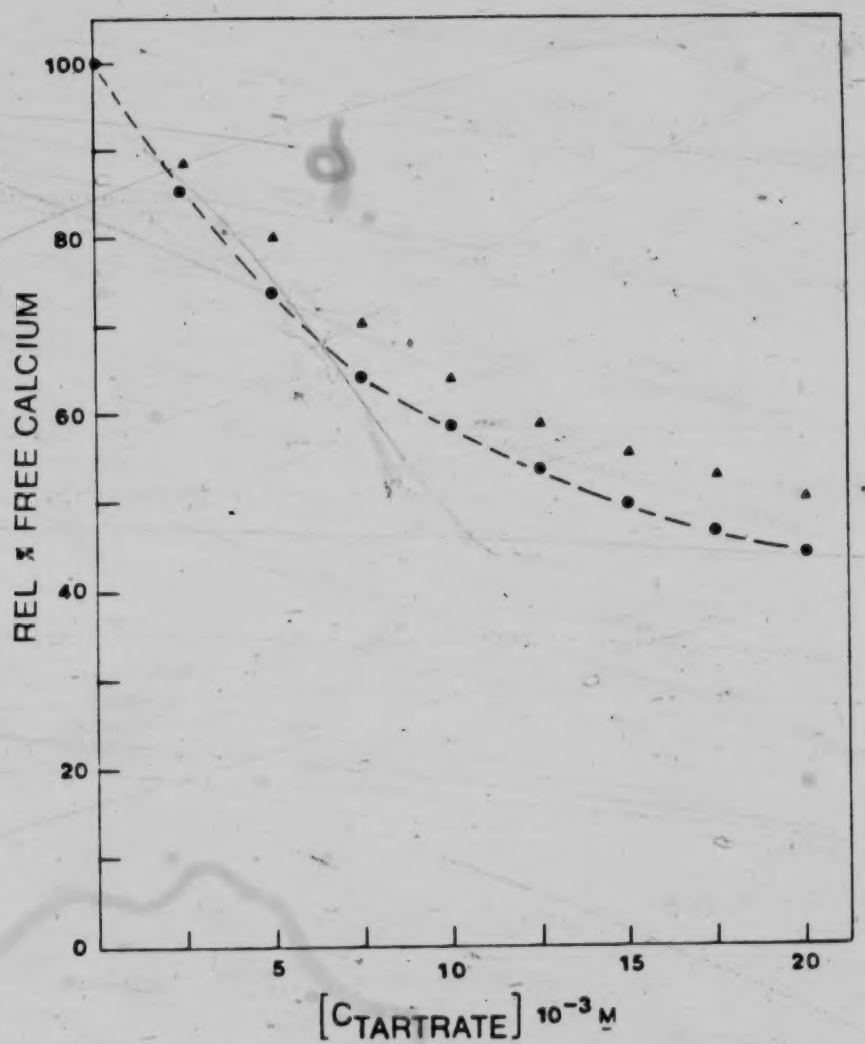


Figure 5.7 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in presence of tartrate

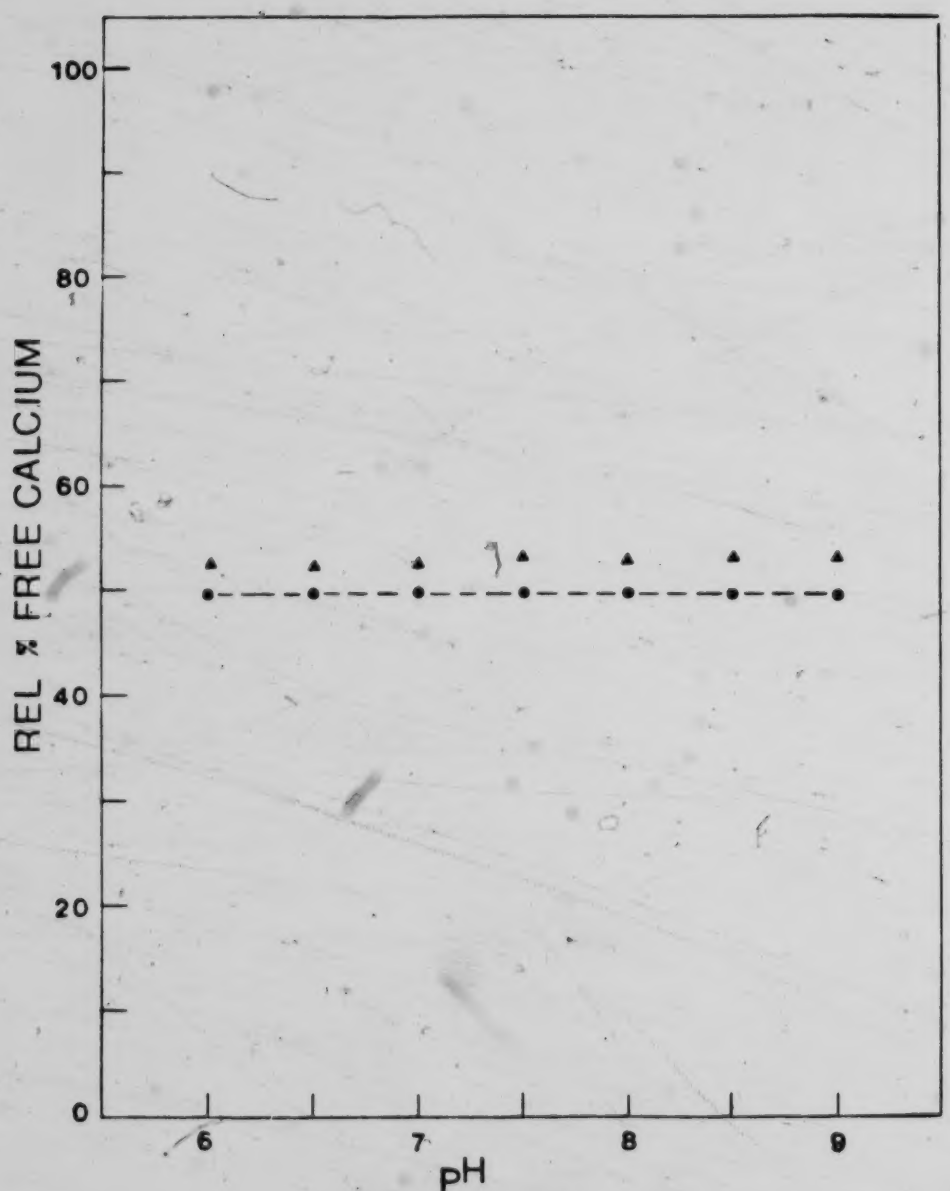


Figure 5.8 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-tartrate system

Effect of Phosphate

These sample solutions contained constant C_{Ca}, 1.00×10^{-5} M as before, and the concentration of the ligand, phosphate, was varied as shown in table 5.2; the ionic strength and the pH were kept constant as before. These sample solutions were loaded on a 5 mg resin column for 11 minutes and were eluted with 3 M HNO_3. The results obtained for the free calcium concentrations, along with calculated values of free calcium obtained from stability constant data for calcium phosphate (140), are plotted in figure 5.9.

A pH study on the calcium phosphate system was also carried out in an attempt to see the effect of pH variation on free calcium concentration. A series of solutions containing a constant C_{Ca} of 1.00×10^{-5} M and C_{PO_4} of 2.00×10^{-4} M were made in which the pH was varied from 6.00 ± 0.05 to 9.00 ± 0.05 in increments of 0.50 ± 0.05 as shown in figure 5.10. This figure shows the value of free calcium obtained experimentally and by calculation using the stability constant data for calcium phosphate and acid dissociation constants of the ligand (140).

Effect of Oxalate

Synthetic solutions contained a constant concentration of C_{Ca}, 1.00×10^{-5} M, and the concentration of added ligand was varied to achieve different degrees of calcium buffering as shown in table 5.2. The pH and ionic strength were held constant as before at 7.04 ± 0.05 and 0.10. The solutions were then loaded onto a 5 mg resin column for 11 minutes and then eluted with 3 M HNO_3. The results obtained for the pCa values obtained experimentally and those obtained by calculation using the stability constant data

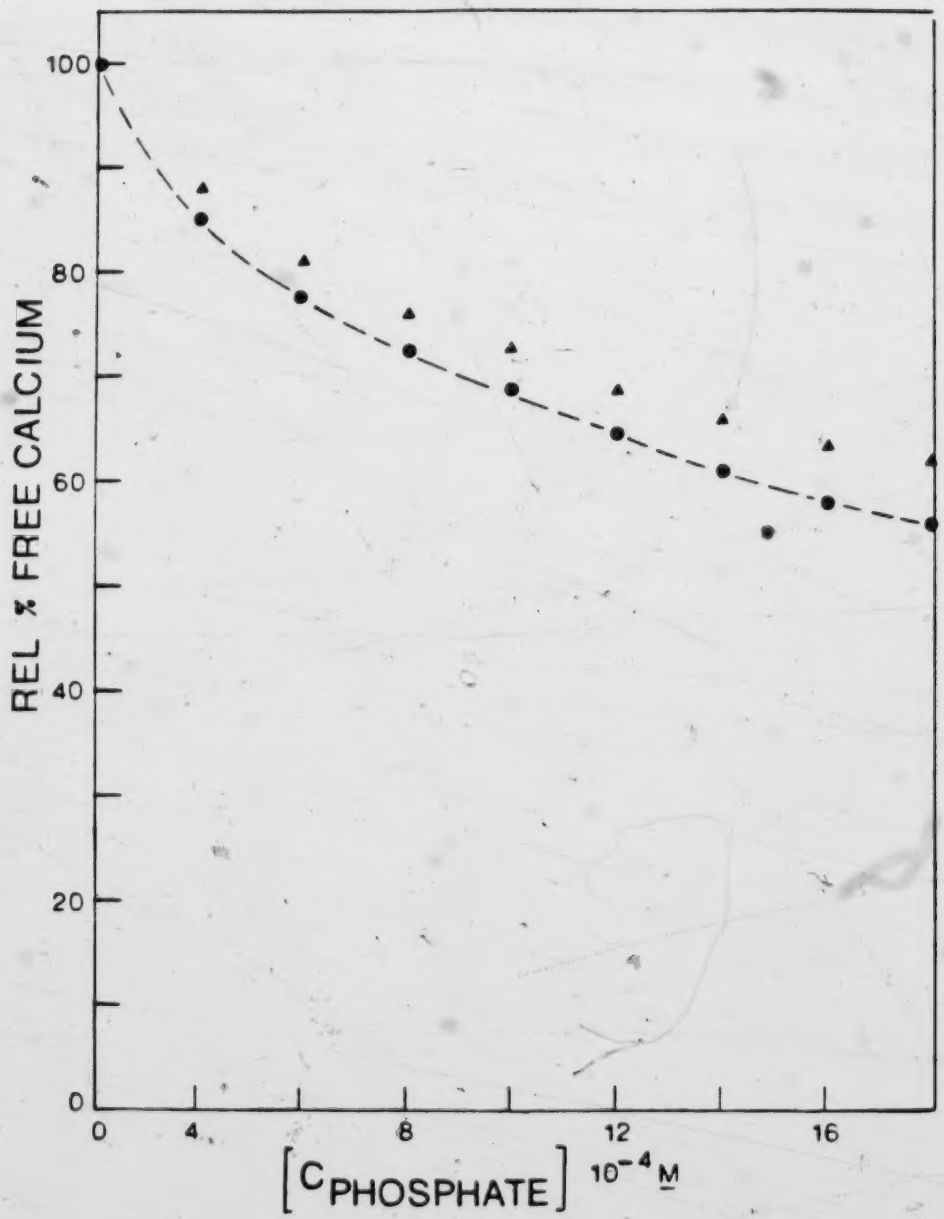


Figure 5.9 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in the presence of phosphate

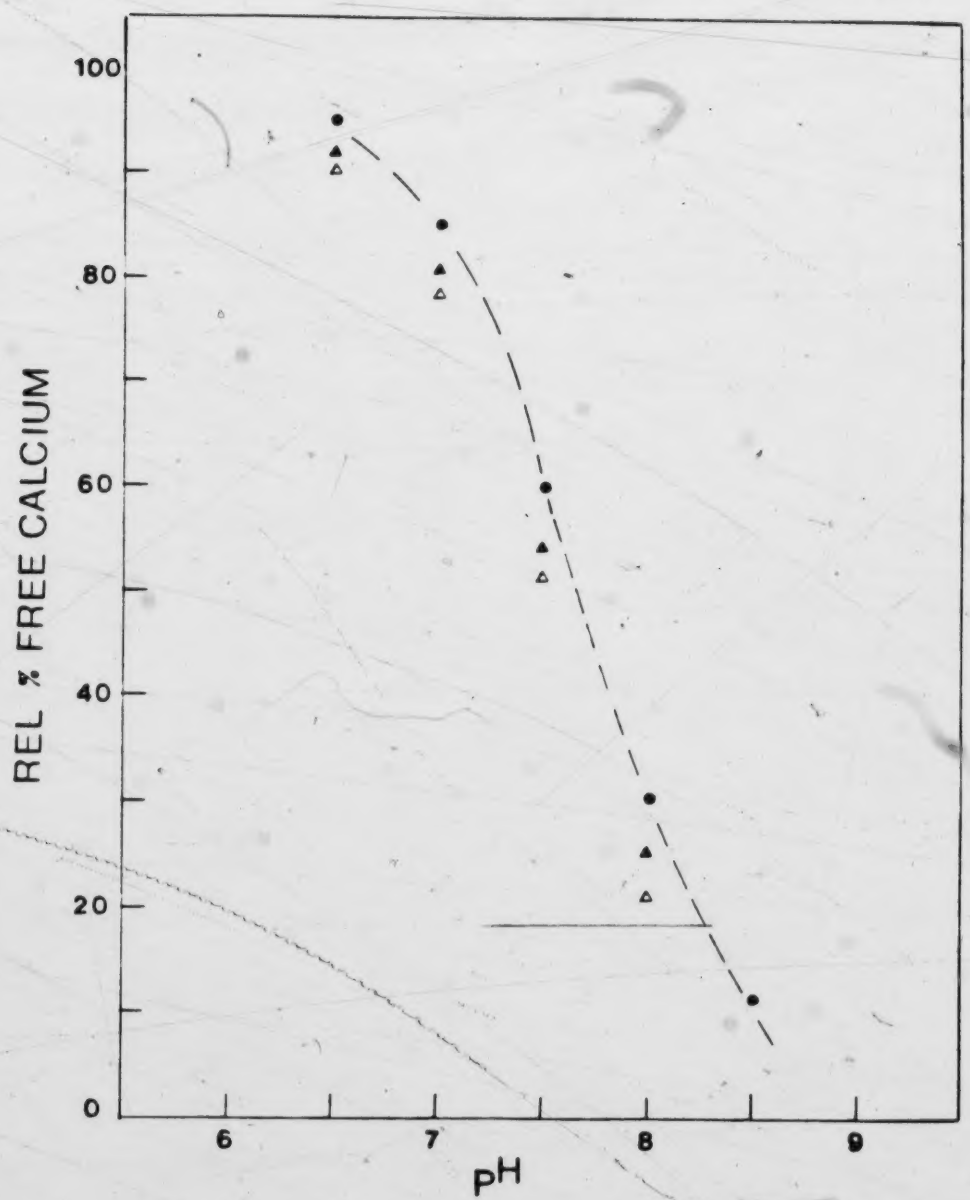


Figure 5.10 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-phosphate system

of calcium oxalate and acid dissociation constant of the ligand (140) are shown in figure 5.11.

A pH variation study was also carried out keeping C_{Ca} and $C_{oxalate}$ constant at 1.00 X 10^{-5} M and 8.00 X 10^{-4} M in a series of solutions in which the pH was varied from 6.00 ± 0.05 to 9.00 ± 0.05 in increments of 0.50. Loading of the solutions and analysis were as before. The results obtained for the free calcium concentrations are shown in figure 5.12 along with the values obtained by calculation (140).

Effect of NTA

The concentration of the added ligand NTA was varied and C_{Ca} was kept constant at 1.00 X 10^{-5} M in a series of solutions as shown in table 5.2. These solutions were then analyzed as before by loading onto a 5 mg resin column for 11 minutes and eluting with 3 M HNO_3. Figure 5.13 summarizes the results obtained for the free calcium concentration and the values of pCa calculated using the stability constant data for calcium NTA and the acid dissociation constants of the ligand (140).

A pH variation study on the calcium-NTA system was also carried out by keeping C_{Ca} and C_{NTA} constant at 1.00 X 10^{-5} M and 6.00 X 10^{-7} M and varying the pH as before. The solutions were analyzed as above for free calcium; the results obtained are shown in figure 5.14.

5.4 Discussion

The results of the free calcium concentrations measured in various synthetic samples by the IEE-AAS technique showed that the values of

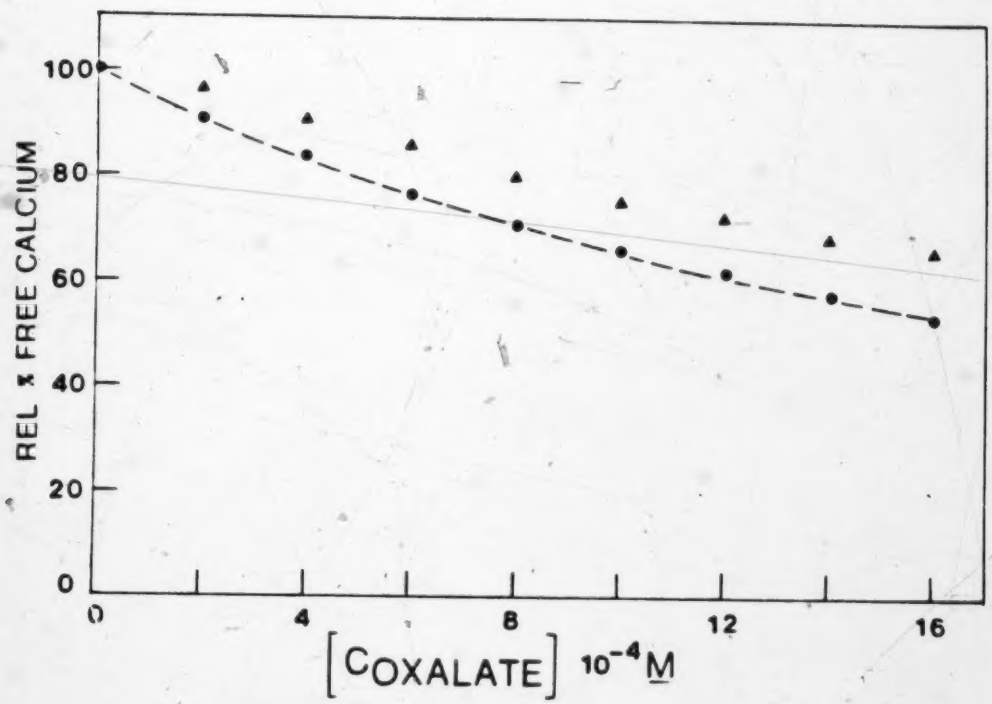


Figure 5.11 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in the presence of oxalate

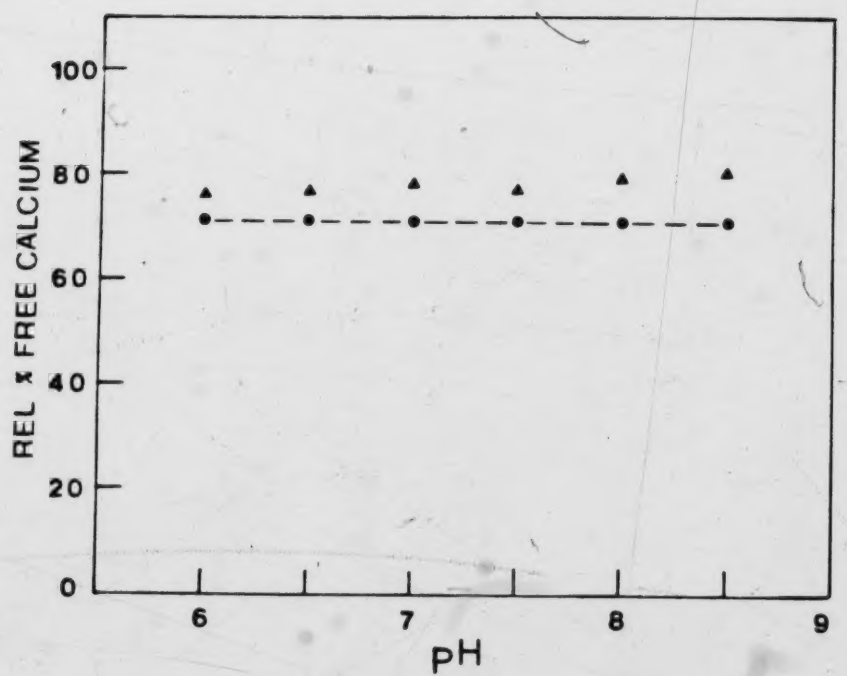


Figure 5.12 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-oxalate system.

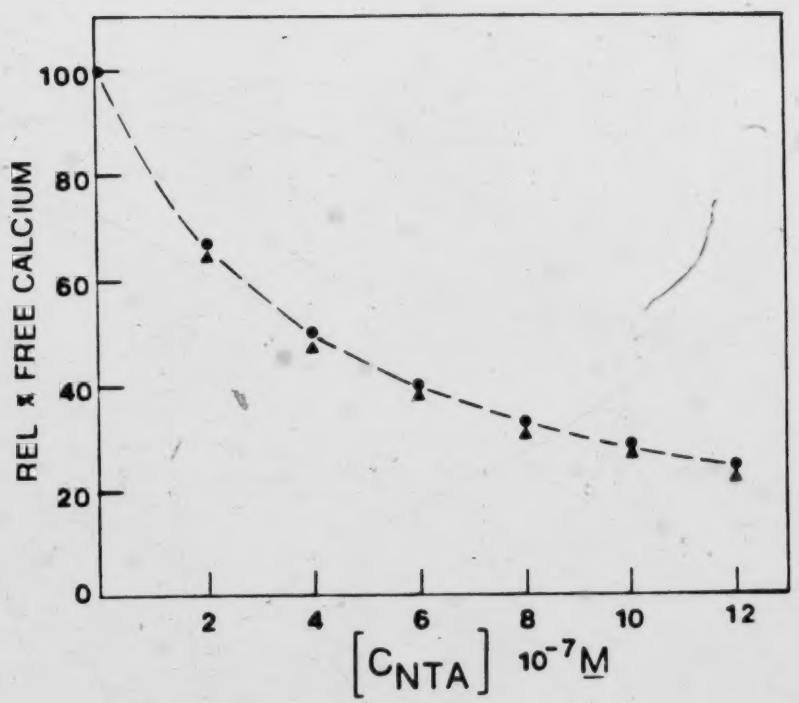


Figure 5.13 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations obtained by IEE-AAS, in the presence of NTA

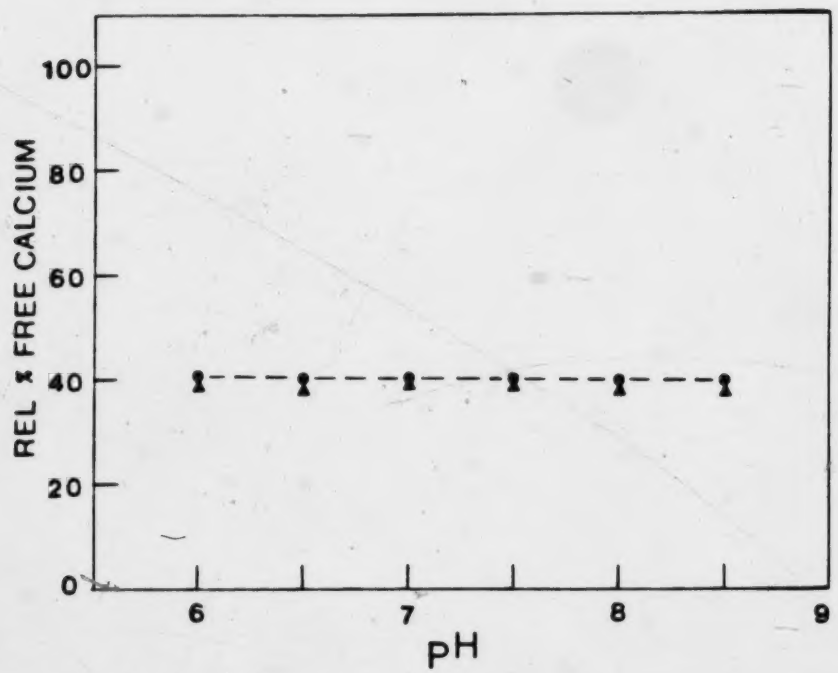


Figure 5.14 Comparison of experimental (▲) and calculated (●) values of free calcium concentrations on varying the pH of calcium-NTA system

pCa obtained experimentally agree with those calculated within 5 to 10% relative error. The discrepancy between pCa_{exp} and pCa_{calc} may be explained in part on the basis of uncertainties in the stability constant values of the calcium-ligand species and the acid dissociation constant values of the ligand under consideration.

The higher value of pCa_{exp} obtained in the case where citrate, tartrate or oxalate is present might be explained by the presence of a neutral species [CaL] which may be absorbed by the cation exchange resin and be eluted along with the free calcium, thereby contributing a higher free ion value for calcium than is actually present. Retention of neutral species by cation exchange resins has been shown before (141). The fraction of cationic species as $[CaH\ Tart]^+$ is too low to influence results under the conditions of this experiment. The formation of colloidal calcium oxalate when oxalic acid was present in the sample might cause higher values of pCa_{exp}, since the colloidal calcium oxalate could be trapped on the resin and be eluted along with the free calcium on elution with HNO_3.

The effect of pH variation on the calcium-oxalate, calcium-NTA and calcium-tartrate systems is neglible and is as predicted (Figures 5.8, 5.12 and 5.14) in the pH range studied. Since on varying the pH the fraction of the unprotonated (free) ligand does not vary for these systems, no effect was seen in the values of pCa obtained by experiment. The free concentration of calcium did change on varying the pH of the calcium-citrate and calcium - phosphate systems considerably as expected (Figures 5.6 and 5.10), since the fraction of free ligand varies on varying the pH and hence significantly changes the free calcium concentration.

5.5 Determination of Free Calcium Concentrations in Urine:

Preliminary studies using the ion-exchange equilibrium method were carried out for the determination of free calcium in urine samples. The urine samples were obtained from the University of Alberta Hospital, and were analyzed within 24 hours to avoid sample decomposition and bacterial growth. Although these problems can be avoided by the addition of preservatives to the samples, it was decided to do the analyses immediately, since addition of preservatives might perturb existing equilibria in the sample. The urine samples were centrifuged prior to analysis to remove suspended particles present, which might clog the resin on loading the sample. The pH values of the urine samples were 6.17 ± 0.05, 6.31 ± 0.05, 6.26 ± 0.05, 6.01 ± 0.05 and 6.11 ± 0.05 for samples 1 to 6. The first four samples contained more than 300 mL, hence replicate analysis was possible; the last two samples (5 and 6) were 140 and 158 mL and allowed only a single measurement of each.

The urine samples were loaded onto a 5 mg resin column for two different time periods, 11 and 14 minutes, to ensure equilibration has been reached between the resin and the sample solution. After the loading step the column was washed twice with wash solution (30% methanol + 70% deionised water) to remove any neutral species present in the column and then the calcium was eluted with 3 M HNO_3 directly into an atomic absorption spectrophotometer. From the two different loading times it was observed that the signal did not change on increasing the loading time from 11 to 14 minutes; hence all other samples were analyzed using a loading time of 11 minutes. The calibration for the IEE-AAS consisted of running a series of calcium standard solutions in which the ionic strength was adjusted to 0.20 with NaCl to approximately match the samples. The equilibration times on loading the solutions

whose ionic strength was adjusted with NaCl did not vary significantly from those in which KNO_3 was used to adjust the ionic strength. Equilibration times of 11 minutes with KNO_3 and 10.2 minutes with NaCl were obtained for a column containing 5 mg resin.

Measurements were also carried out using a calcium ion-selective elecrtode in this study. The calcium ion selective electrode was calibrated with standard calcium solutions in which the ionic strength was adjusted to 0.20 with NaCl. These solutions were prepared in the same way as before for the IEE-AAS studies.

5.5.1 Results and Discussion:

The ionic strength of urine varies widely, and at the present time no accurate and convenient method is available for its determination. Hunt and King (137) estimated the ionic strength of urine samples by comparing their conductance with standards prepared from potassium chloride. However, the relationship between conductance and ionic strength has been shown to be only an approximation (138). Other workers have estimated the ionic strength of urine samples from measurements of major cations present in the sample (139), but this approach is tedious and once again gives only approximate results.

The ionic strength of the urine was estimated using literature values for the most abundant ionic species normally present. These are usually reported as a range of values rather than a single value. The maximum and the minimum individual ionic strengths were summed to give the total maximum and minimum ionic strength. With this approach the range of ionic strengths obtained were 0.16 minimum and 0.45 maximum (49); this range should not be taken as a reference because ionic strength will vary depending upon the diet of a patient.

The activity coefficient for calcium ion was calculated using the extended Debye-Huckel equation;

$$- \log f_{Ca} = 0.5 z_i^2 \sqrt{\mu} / (1 + \sqrt{\mu})$$

For $\mu = 0.16$: fCa = 0.27

$\mu = 0.20$: fCa = 0.21

$\mu = 0.45$: fCa = 0.16

From the above calculation it is clear that a considerable error will be introduced if the ionic strength of the urine sample is not known with reasonable accuracy, since the concentration is directly related to the activity and any variation in the activity of calcium in the sample will introduce error. Hence the measurement of the free concentration of the calcium by potentiometry using the ion - selective electrode is subject to some error.

The results obtained for the free calcium concentration in the various urine samples are summarized in table 5.3.

The free calcium concentration values obtained by the IEE-AAS technique are also subject to error owing to two unknown parameters in the system. However, the values obtained are still in the range reported in the literature (31-32). The calcium in sample 1, 2, and 4 was too small to be measured with the ion selective electrode; i.e. it was at the limit of detection, approximately 2 mg/L.

Although one can determine the concentration of Na^+ in urine samples by flame photometry before analyzing for free calcium by IEE-AAS, the second parameter, ionic strength, still remains unknown. Therefore the method after adjustment of the sodium ion concentration

Table 5.3

Camparision of Ion-Exchange Equilibration / Atomic Absorption and Ion Selective Electrode for the Determination of Ionic Calcium in Urine Samples :

Sample #		Ionic Ca (mg / L)	
	pH	Ion Selective Electrode	Ion-Exchange
1	6.17±0.05	< 2	2.68 *
2	6.31±0.05	< 2	1.05 *
3	6.26±0.03	5.23	4.41 *
4	6.21±0.03	< 2	2.26 *
5	6.01±0.05	7.11	6.2
6	6.12±0.05	7.78	6.7

*Average of 3 runs ; other values are single measurements.

will still give only approximate results which will have some errors as before but of smaller magnitude. It can be concluded that the IEE-AAS method for measurement of ionic calcium in urine shows promise, and is worth further study, especially if a method for the rapid estimation of the sodium concentration and the ionic strength were available.

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